Metabolic engineering of lactic acid bacteria and characterization of novel enzymes for the production of industrially important compounds

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ACADEMIC DISSERTATION

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ABSTRACT

Lactic acid bacteria (LAB) are a heterogeneous group of gram-positive bacteria that produce lactic acid as their main end-product during sugar fermentation. Because the LAB are able to rapidly lower pH through acid formation and additionally produce many flavor compounds, they are commonly used in the food and feed industry. LAB are also attractive organisms for metabolic engineering because their energy metabolism is generally not connected to their biosynthetic activity. Therefore, their sugar metabolism can be engineered without substantial interference to the biosynthesis pathways. This engineering ability has increased the interest in and significance of LAB as novel hosts for the production of valuable metabolites.

During the past decade, tools for genetic modification of LAB have also been developed and considerable advancements in metabolic engineering of LAB have been made. For example, metabolic engineering of the pyruvate metabolism of LAB has resulted in efficient production of diacetyl and L-alanine. In addition, important advances have been made in the metabolic engineering of more complex biosynthetic pathways leading to products such as exopolysaccharides and vitamin B.

In this work, the main target was to modify the metabolism of LAB to produce industrially important compounds. Therefore, the first part of this work describes metabolic engineering to improve the production of mannitol, pyruvate and L-lactic acid in LAB. The second part describes isolation, cloning and characterization of genes encoding enzymes that are promising for the further development of genetically modified lactic acid bacteria.

In the first part, either the *ldhD* and *ldhL* genes, or *ldhD* alone, were inactivated using gene replacement techniques from a *Lactobacillus fermentum* strain known to be an efficient mannitol producer. With these gene inactivations two *L. fermentum* mutant strains were constructed, which produce mannitol and either pure L-lactate or pyruvate in a single process. A successful improvement of mannitol production by *Leuconostoc pseudomesenteroides* was also achieved by using random mutagenization to decrease its fructokinase activity. This would diminish the leakage of fructose from mannitol synthesis to the catabolic sugar pathway. Furthermore, the gene encoding the fructokinase and its putative promoter region were characterized.

The mannitol dehydrogenase (MDH) enzyme has a central role in producing mannitol from fructose in heterofermentative LAB. Therefore, in the second part of this work, the mannitol dehydrogenase gene (*mdh*) of *Leuconostoc mesenteroides* was isolated and overexpressed in *E. coli* to characterize its enzymatic properties. This was the first *mdh* gene characterized from heterofermentative LAB and it was later confirmed that *mdh* genes from this group of bacteria are clearly distinct from other bacterial *mdh* genes. Furthermore, we cloned a novel xylitol-4-dehydrogenase gene (*xdh*) of the gram-negative *Pantoea ananatis*. This is the first available sequence of a bacterial gene, which encodes an enzyme catalyzing the oxidation of xylitol to L-xylulose. This XDH enzyme offers an interesting opportunity to produce L-xylulose, for example, in recombinant LAB strains.

ABBREVIATIONS

ALDB	α-acetolactate decarboxylase
ALR	alanine racemase
ALS	α-acetolactate synthase
ATP	adenosine triphosphate
bp	base pair
CcpA	catabolite control protein A
cDNA	single stranded DNA, complementary to RNA
cre	catabolite responsive element
DHAP	dihydroxyacetonephosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
FDP	fructose-1,6-diphosphate
FK	fructokinase
GAP	glyceraldehyde-3-phosphate
HPLC	high-performance liquid chromatography
HPr	heat-stable phosphocarrier protein
ILVBN	acetohydroxy acid synthase
IPTG	isopropyl-β-D-thiogalactopyranoside
IR	inverted repeats
IS	insertion sequence
kDa	kilo Dalton
K_m	Michaelis constant
LAB	lactic acid bacteria
L-AlaDH	L-alanine dehydrogenase
LDH	lactate dehydrogenase
MDH	mannitol dehydrogenase
MDR	medium chain dehydrogenase/reductase superfamily
MLF	malo-lactic fermentation
MMS	methyl methanesulfonate
MNNG	N-methyl-N-nitro-N´-nitrosoguanidine
MPDH	mannitol 1-phosphate dehydrogenase
MP	mannitol 1-phosphate phosphatase
mRNA	messenger RNA
NAD^+/NADH	oxidized/reduced form of nicotinamide adenine dinucleotide
$NADP^+$/NADPH	oxidized/reduced form of nicotinamide adenine dinucleotide phosphate
NICE	nisin controlled expression system
NMR	nuclear magnetic resonance
NOX	NADH oxidase
ORF	open reading frame
ori	origin of replication
PaseII	sugar-P phosphatase
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate

PGI	phosphoglucose isomerase
6-PG/PK	6-phosphogluconate/phosphoketolase
P_i	inorganic phosphate
PMF	proton motive force
POX	pyruvate oxidase
PTS	phosphotransferase system
RepA	replication initiation protein
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SDH	sorbitol dehydrogenase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHMT	serine hydroxymethyltransferase
UV	ultraviolet (radiation)
XDH	xylitol-4-dehydrogenase

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following puclications referred to in the text by their Roman numerals:

I. Aarnikunnas J, von Weymarn N, Rönnholm K, Leisola M, Palva A. 2003. Metabolic engineering of *Lactobacillus fermentum* for production of mannitol and pure L-lactic acid or pyruvate. Biotechnol Bioeng. 82:653-63.

II. Aarnikunnas J, Rönnholm K, Palva A. 2002. The mannitol dehydrogenase gene (*mdh*) from *Leuconostoc mesenteroides* is distinct from other known bacterial *mdh* genes. Appl Microbiol Biotechnol. 59:665-71.

III. Helanto M, Aarnikunnas J, von Weymarn N, Airaksinen U, Palva A, Leisola M. 2005. Improved mannitol production by a random mutant of *Leuconostoc pseudomesenteroides*. J Biotechnol. 116:283-94.

IV. Aarnikunnas J, Pihlajaniemi A, Palva A, Leisola M, Nyyssölä A. 2006. Cloning and expression of a xylitol-4-dehydrogenase gene from *Pantoea ananatis*. Appl Environ Microbiol. 72:368-77.

1. INTRODUCTION

1.1. Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of bacteria characterized by their ability to synthesize lactic acid. Typical LAB are gram-positive, nonsporing, catalase-negative, devoid of cytochromes, anaerobic but aerotolerant cocci or rods that are acid-tolerant and produce lactic acid as the major end product during sugar fermentation (Axelsson 2004). However, under certain conditions some LAB do not display all these characteristics. Thus, the most profound features of LAB are gram positiveness and an inability to synthesize porphyrin groups.

The LAB could be comprised of about 20 genera. The genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, C*arnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus* and *Weisella* are the main members of the LAB (Axelsson 2004, Davidson et al. 1995). *Lactobacillus* is largest of these genera, comprising around 80 recognized species (Axelsson 2004).

Traditionally, the LAB have been widely used as starter bacteria in the fermentation of milk, meat, fish and vegetable products. These food products have a low pH as a result of excreted lactic acid, which hinders the growth of various food spoilage and pathogenic organisms (Davidson et al. 1995). The LAB are also normal inhabitants of the mucosal surfaces of humans and animals (Axelsson 2004).

The inability to synthesize porphyrin (e.g., heme) results in the LAB being devoid of catalase and cytochromes (without supplemented heme in the growth media). Therefore, the LAB do not possess an electron transport chain and rely on fermentation to generate energy (Axelsson 2004). The LAB have two main hexose monosaccharides fermentation pathways, which are homolactic fermentation, in other words glycolysis (Embden-Meyerhof-Parnas pathway) and heterolactic fermentation, i.e. the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway. Based on these two main fermentation pathways the LAB have been divided into three metabolic categories: obligate homofermentative, obligate heterofermentative and facultative heterofermentative. The obligate homofermentative LAB can only ferment sugars by glycolysis, while the obligate heterofermentative LAB use only the 6-PG/PK pathway and the facultative heterofermentative LAB have the capability to utilize both pathways.

1.2. Glycolysis (Embden-Meyerhof-Parnas pathway)

In glycolysis (Embden-Meyerhof-Parnas pathway), under normal conditions where sugars are not limiting and oxygen is confined, one glucose molecule is theoretically fermented to two lactic acid molecules resulting in a net gain of two molecules of ATP (Figure 1). The first steps of glycolysis are the phosphorylation of glucose to fructose-1,6-diphosphate (FDP) and its splitting into dihydroxyacetonephosphate (DHAP) and glyceraldehyde-3-phosphate (GAP), (DHAP formed is also converted to GAP). GAP is then converted to pyruvate via a route that includes two substrate-level phosphorylation

steps. Finally, pyruvate is reduced to lactic acid by lactate dehydrogenase (LDH) using NADH as the cofactor. In glycolysis the reduced cofactors NADH are reoxidized to NAD^+ and thus a redox balance is obtained (Axelsson 2004).

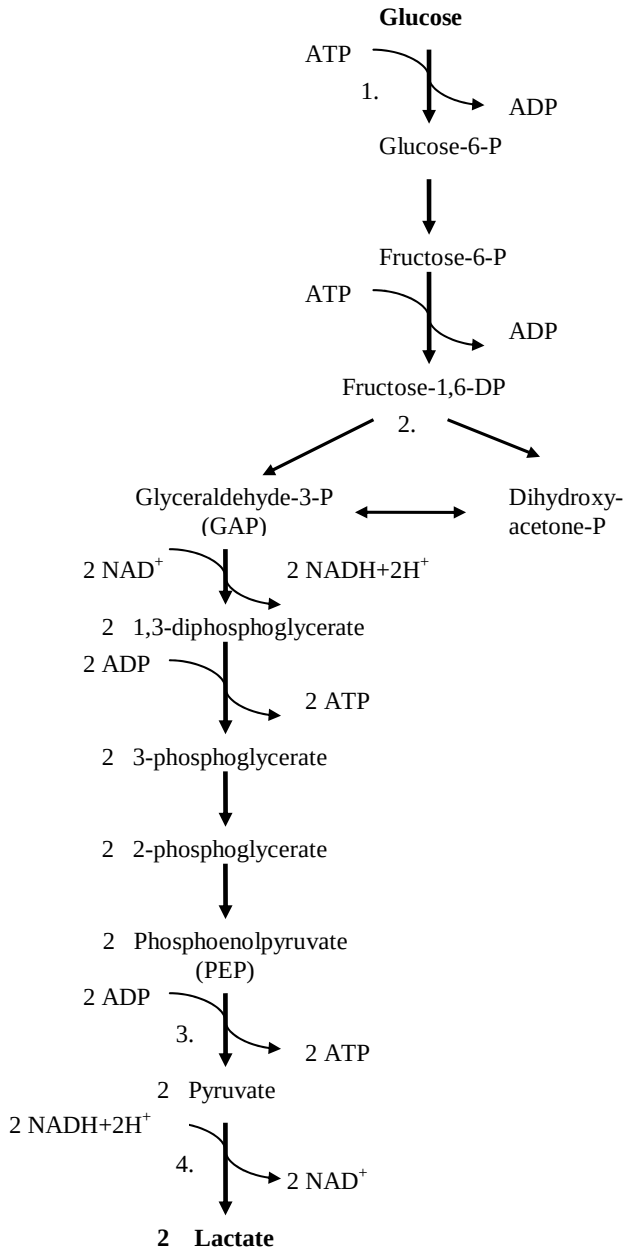

Figure 1. Glycolysis (Embden-Meyerhof-Parnas pathway). The enzymes: 1. glucokinase, 2. fructose-1,6-diphosphate aldolase, 3. pyruvate kinase and 4. lactate dehydrogenase. Adapted from Axelsson (2004)

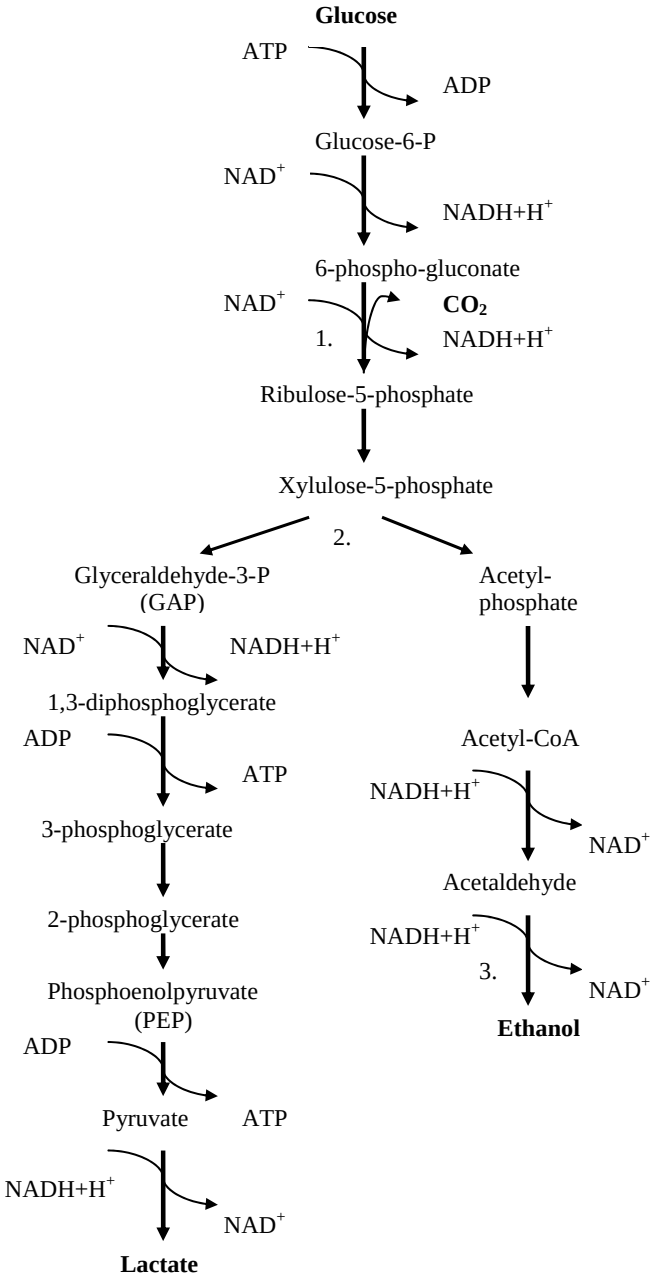

Figure 2. The 6-phosphogluconate/ phosphoketolase (6-PG/PK) pathway. The enzymes: 1. 6-phospho-gluconate dehydrogenase, 2. phosphoketolase, and 3. alcohol dehydrogenase. Adapted from Axelsson (2004).

1.3. 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway

In the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway, lactic acid is not the only end product; in addition, CO_2 and ethanol are produced, and the 6-PG/PK pathway

is hence also named heterolactic fermentation (Figure 2). In the 6-PG/PK pathway, the theoretical net gain of ATP is one mol ATP/mol glucose, which is only half of that obtained in glycolysis.

The first phosphorylation step of glucose in the 6-PG/PK pathway is the same as in glycolysis. The key pathway steps are the dehydrogenation of glucose-6-P to 6-phosphogluconate, its decarboxylation followed by the splitting of xylulose-5-phosphate into glyceraldehyde-3-phosphate (GAP) and acetyl-phosphate by phosphoketolase. GAP is metabolized to lactic acid via the same pathway as in glycolysis. Without an additional electron acceptor, acetyl-phosphate is in turn reduced to ethanol via acetyl CoA and acetaldehyde (Axelsson 2004).

1.4. Fermentation of pentoses

Pentoses are transported into cells by specific permeases and subsequently phosporylated inside the cells to ribulose-5-phosphate or xylulose-5-phosphate by isomerases or epimerases. The metabolism of these compounds continues in the lower part of the 6-PG/PK pathway. This heterolactic fermentation of pentoses results in a different end product pattern compared to glucose fermentation. No CO_2 is formed, and since the dehydrogenation steps are unnecessary for reaching the intermediate xylulose-5-phosphate, there is no reduction of acetyl phosphate to ethanol to maintain a redox balance. Instead, ATP and acetate are formed from acetyl phosphate by acetate kinase (Axelsson 2004).

Few species of LAB (*Streptococcus avium* and *Lactobacillus casei*) can utilize pentitols e.g. ribitol, xylitol and D-arabitol. These pentitols are imported into the cell and converted to their respective pentitol-5-phosphates by a substrate-specific phosphotransferase system (PTS) (see chapter 1.8.3.). The formed pentitol phosphates are oxidized to pentose phosphate by NAD^+ dehydrogenases and then enter the 6-PG/PK pathway (London 1990).

1.5. Co-metabolism

The heterofermentative LAB can form mannitol using fructose both as an electron acceptor and a growth substrate (Figure 3). Part of the fructose is reduced to mannitol by mannitol dehydrogenase and part is phosphorylated by fructokinase and isomerased to glucose-6-P, which is fermented normally in the 6-PG/PK pathway. The net equation of fructose fermentation is:

3 fructose + 2 ADP + 2 P_i à lactate + acetate + CO_2 + 2 mannitol + 2 ATP

The net gain of ATP/ mol sugar is not as efficient as in glucose fermentation, but under conditions of substrate excess the cells may be able produce more ATP per time unit. In conditions where both these sugars exist, the heterofermentative LAB can optimize their growth rate using glucose as an energy source and fructose as an electron acceptor (Axelsson 2004).

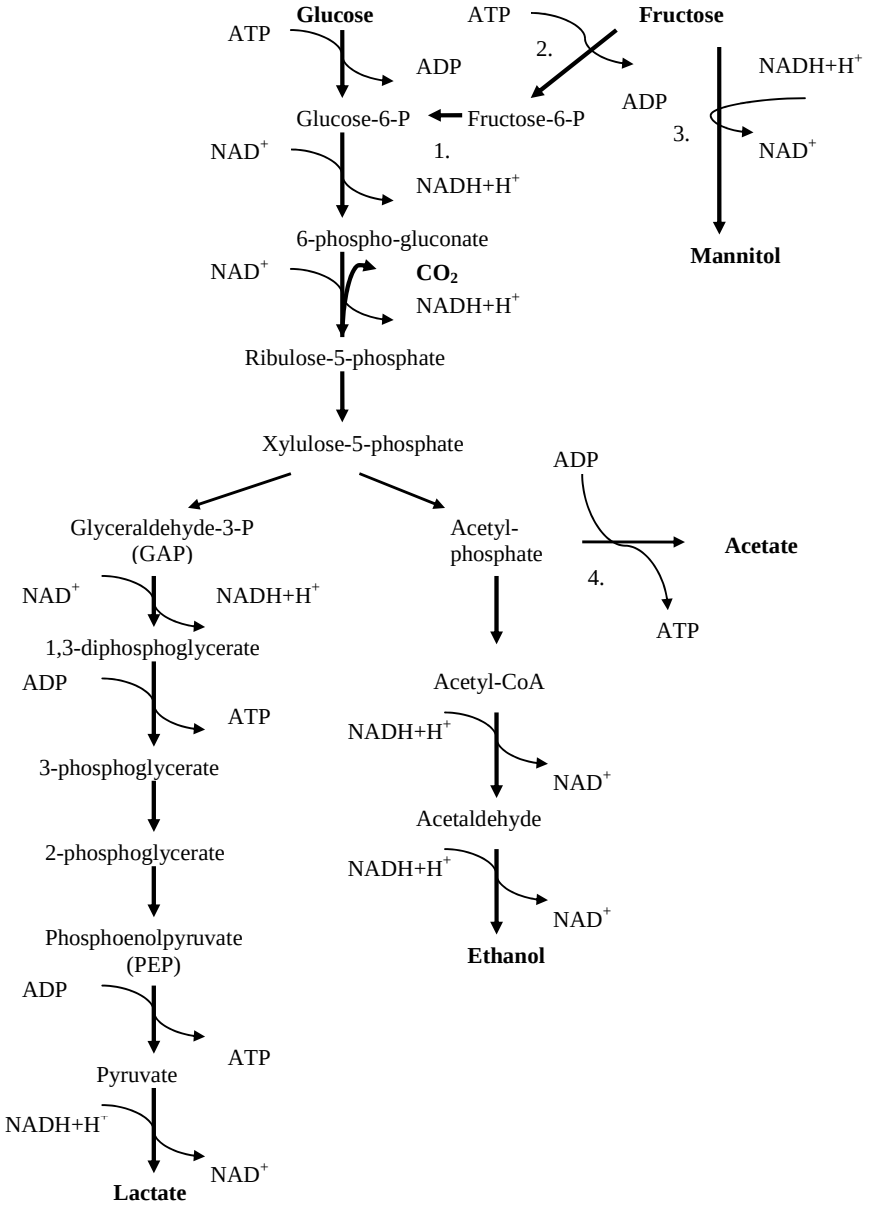

Figure 3. Co-metabolism of fructose and glucose. The enzymes: 1. phosphoglucoisomerase, 2. fructokinase, 3. mannitol dehydrogenase and 4. acetate kinase. Adapted from Axelsson (2004).

1.6. Malo-lactic fermentatation

L-malic acid (malate) is a common compound in fruits and plants (Arthurs & Lloyd 1999, Pilone et al. 1966). Some LAB can use malate as their sole energy source. These LAB have a malic enzyme that catalyzes the decarboxylation of malate to pyruvate and CO_2 using NAD^+ as a cofactor. A more common pathway for malate dissimilation in

LAB is referred to as malo-lactic fermentation (MLF). This is not actually a correct term, since the transformation of L-malic to L-lactic acid is a decarboxylation reaction, not a fermentative pathway (Lonvaud-Funel 1999). However, in MLF malate is converted directly to lactate and CO_2 by the malo-lactic enzyme (L-malate NAD^+ carboxylase) (Caspritz & Radler 1983, Lonvaud-Funel 1999).

The malate is transported into the cells by secondary transporters (see chapter1.8.3.), or when the malate concentration is high it may enter by diffusion (Bandell et al. 1997, Olsen et al. 1991, Salema et al. 1996). Inside the cell, malate is converted to lactate, which is exported with a proton out of the cells, thus creating an electrochemical proton gradient across the cell membrane. The developed proton gradient in turn drives ATP synthesis (Olsen et al. 1991, Salema et al. 1996).

1.7. Different pathways of pyruvate

Pyruvate has an essential role in the metabolism of LAB serving as an electron acceptor, which enables the regeneration of NAD^+ in order to continue fermentation. Depending on the circumstances, pyruvate can be utilized in alternative ways other than reduction to lactate (Figure 4). The ability to use these different pyruvate pathways is strain specific.

1.7.1. Diacetyl/acetoin pathway

Diacetyl and acetoin/2,3-butanediol are the end products in the diacaetyl/acetoin pathway (Figure 4). The pathway proceeds only if there is a pyruvate surplus in the cell relative to the need for NAD^+ regeneration. This situation is possible if the source of pyruvate, other than the fermented carbohydrate, exists in the growth medium or another compound acts as the electron acceptor instead of pyruvate (e.g. oxygen). In general, low sugar concentrations and a low pH favor diacetyl/acetoin formation (Axelsson 2004). α-Acetolactate is an intermediate formed from pyruvate by acetolactate synthase. Diacetyl is nonenzymatically formed by chemical decomposition of α-acetolactate, and this reaction is favored by low pH and aeration. In this pathway, acetoin and/or 2,3-butanediol are produced in excess of diacetyl (Axelsson 2004).

1.7.2. Pyruvate-formate lyase system

Pyruvate-formate lyase catalyzes the formation of acetyl CoA and formate from pyruvate and CoA (Figure 4). The acetyl CoA can then serve either as an electron acceptor, resulting in ethanol formation, or as a precursor of ATP formation, resulting in acetate. The pyruvate-formate lyase system is oxygen sensitive and is inactivated in aerobic conditions (Axelsson 2004). In certain LAB (e.g. *L. casei, L. lactis*), under substrate limitations, the pyruvate-formate lyase system is activated, resulting in a change from homolactic to heterolactic fermentation (mixed acid fermentation). The end products are lactate, acetate, formate and ethanol. (Axelsson 2004)

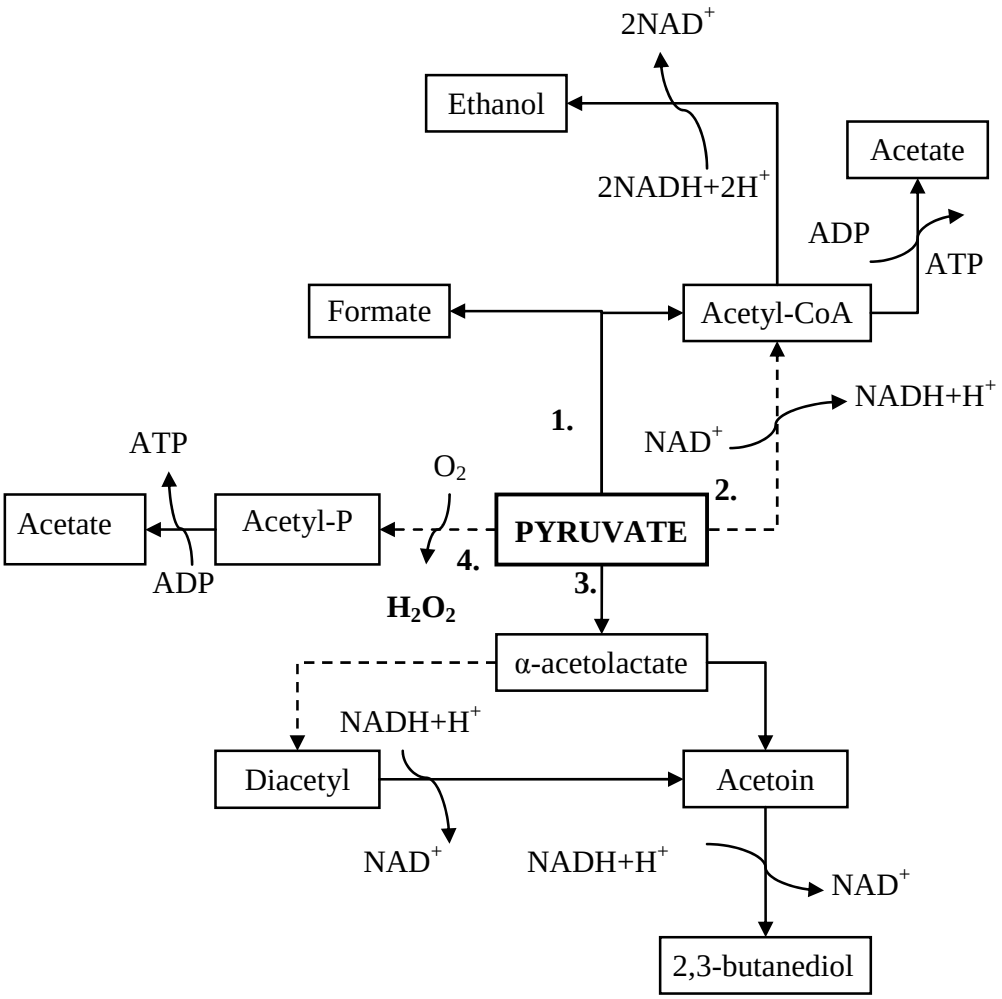

Figure 4. Schematic representation of alternative pathways for pyruvate. 1. Pyruvate-formate lyase system. 2. Pyruvate dehydrogenase pathway. 3. Diacetyl/acetoin pathway 4. Pyruvate oxidase pathway. Dashed lines illustrate the pathways that are favored by aeration. Adapted from Axelsson (2004).

1.7.3. Pyruvate oxidase pathway and NADH oxidases

In LAB, oxygen has a profound effect on the rerouting of pyruvate, being mediated directly by pyruvate oxidase (POX) or indirectly by NADH oxidases (NOX) (Axelsson 2004). Pyruvate oxidase uses oxygen to convert pyruvate into acetyl phosphate, CO_2 and H_2O_2 (Figure 4). The enzyme has the main role in the aerobic formation of acetic acid in *Lactobacillus plantarum* (Lorquet et al. 2004, Sedewitz et al. 1984). The pyruvate oxidase activity in *L. plantarum* is enhanced by oxygen or hydrogen peroxide and is reduced by glucose (Lorquet et al. 2004, Sedewitz et al. 1984).

In many LAB the NADH oxidases are important in controlling the $NADH/NAD^+$ balance in the cell. An alteration in the $NADH/NAD^+$ balance could lead in significant changes in the metabolic routes (Garrigues et al. 1997, Hols et al. 1999a,b, Lopez de Felipe et al. 1998). In the NADH oxidase reaction, oxygen acts as an electron acceptor and NADH is converted to NAD^+ with the formation of H_2O_2 or H_2O. NADH peroxidase also produces NAD^+ and H_2O, but uses H_2O_2 as an electron acceptor (Axelsson 2004).

1.7.4. Pyruvate dehydrogenase pathway

Pyruvate dehydrogenase has an anabolic role under aerobic conditions, producing acetyl CoA for lipid synthesis (Axelsson 2004). In addition, this enzyme complex can have a catabolic role similar to pyruvate-formate lyase, producing acetyl CoA but under aerobic conditions (Figure 4). In this case, acetyl CoA can be metabolized further to acetate with the concomitant formation of ATP. The excess NADH formed in the pyruvate dehydrogenase catalyzed reaction can be reoxidized by NADH oxidases (Axelsson 2004).

1.8. Transport systems and bioenergetics

1.8.1. Bioenergetics

The transport of solutes and the bioenergetics of the cell are in general tightly connected. The electrochemical proton gradient across the cytoplasmic membrane is created by cellular metabolism. This gradient is composed of an electrical potential ($\Delta\Psi$) and a proton gradient (ΔpH). These together form an inwardly directed force named the proton motive force (PMF). The LAB have an enzyme named H^+ translocating ATPase, whose main function is to pump protons out of the cell by hydrolyzing ATP. This ATP-consuming reaction forms PMF, which can in turn drive the transport of ions and metabolites. In respiring organisms the same kind of enzyme, ATP synthase, operates into the opposite direction, forming ATP. In this case, the PMF, which is created by an electron transport chain of respiring organisms, drives external protons inside the cells through membrane-located ATP synthase and ATP is formed from ADP and phosphate.

LAB have three major transport systems for sugar uptake: primary transport, secondary transport and group translocation (phosphotransferase system). *L. lactis* has served as the main model organism in the study of transport systems.

1.8.2. Primary transport

The ABC transport systems (ATP-binding cassette transporters) are the most abundant class of primary transport systems. Translocation of substrates is coupled to ATP hydrolysis (Figure 5). This mechanism is not only used to accumulate different solutes and substrates but also to excrete unwanted products (Poolman 2002). Sugars are transported by carbohydrate transport ATPases (Neves et al. 2005).

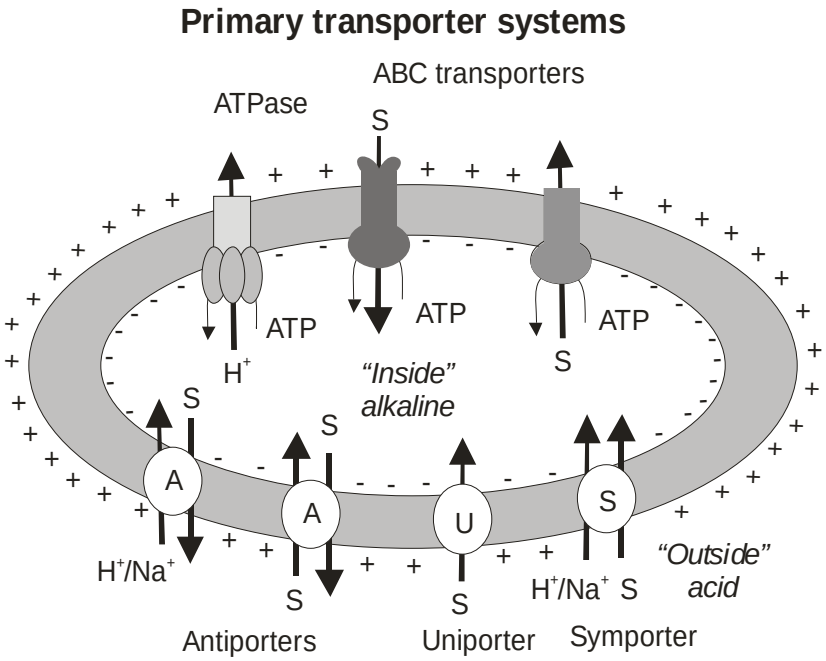

Figure 5. Schematic representation of the primary and secondary transporter mechanisms. S, Na^+ and H^+ refer to solute, sodium and proton, respectively. Adapted from Poolman (2002).

1.8.3. Secondary transport

Secondary transport systems (Figure 5) consist of specific membrane-associated proteins named permeases, which are divided into symporters, uniporters and antiporters. The symporters cotransport two or more solutes, the substrate usually being translocated into the cell with a proton or a sodium ion. In the antiporter system the substrate is excreted from the cell and the proton is driven in the cell. The uniporters do not use a coupling ion (Poolman 2002). Presumably, many sugars are taken up by the cell through symporters via an ion gradient (PMF) and in the cell these free sugars are phosphorylated by kinases (Axelsson 2004, Neves et al. 2005).

1.8.4. Group translocation (phosphotransferase system, PTS)

PTS catalyzes the uptake of sugars across a membrane with a concomitant phosphorylation of the sugars (Figure 6). The PTS is a complex enzyme system where the energy is provided from the phosphate bond of phosphoenolpyruvate (PEP) via the general energy coupling proteins Enzyme I and HPr and the substrate-specific phosphoryl transfer proteins/domains IIA and IIB. Sugar-specific protein components IIA, IIB and IIC can be separate proteins or form the fusion protein named EIIABC (Poolman 2002 Axelsson 2004). PEP is a key compound in the transport of sugars and their subsequent metabolism. PEP can either donate the phosphoryl group to EI and initiate the PTS cycle

or donate it to pyruvate kinase and form ATP (Axelsson 2004). PTS is tightly coupled to the Embden-Meyerhof-Parnas pathway (glycolysis) and is therefore not common in heterofermentative bacteria (Romano et al. 1979, Saier et al. 1996, Taranto et al. 1999).

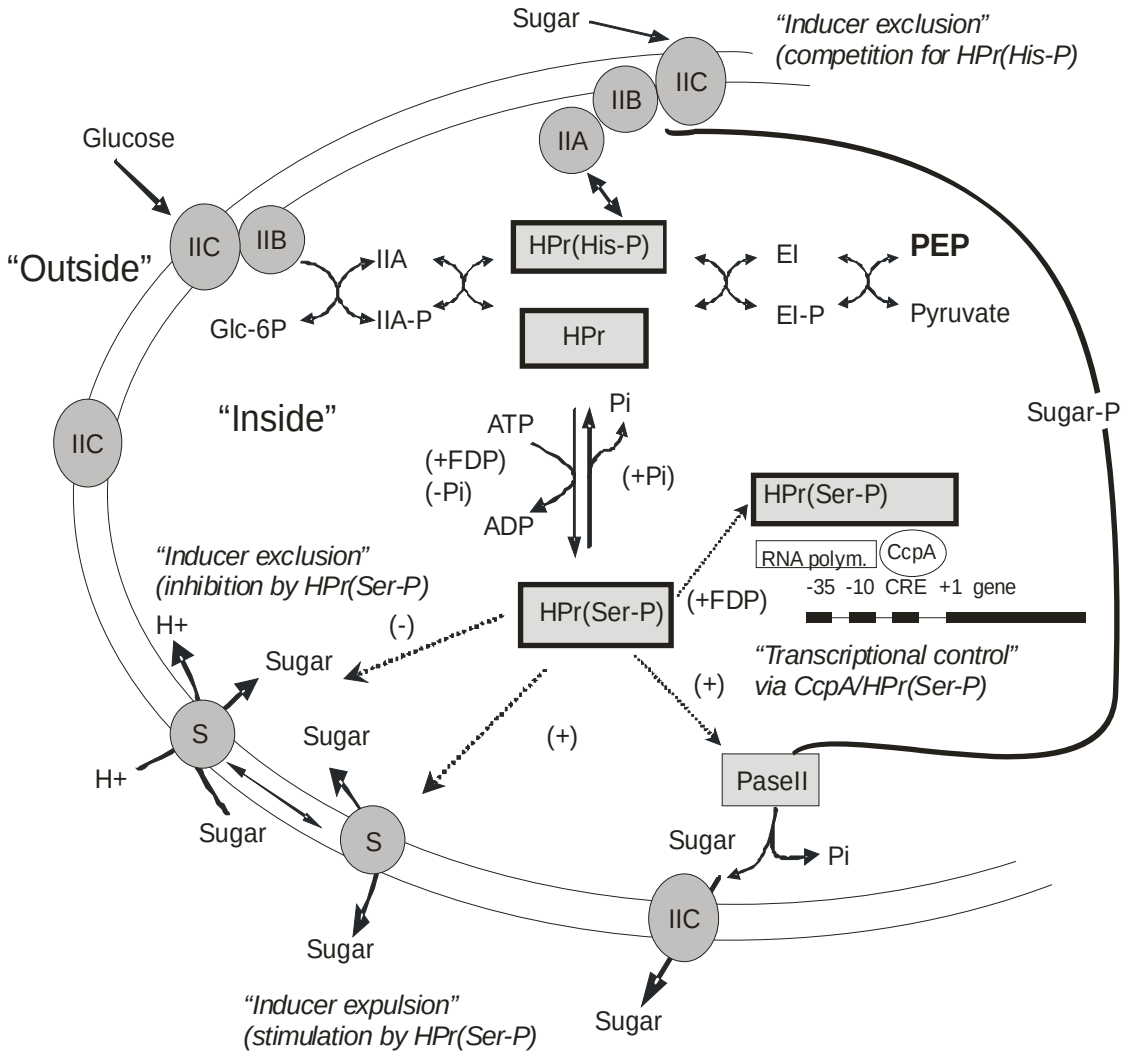

Figure 6. Schematic representation of the phosphotransferase system (PTS) of LAB. The central role of the HPr species in controlling transcription, inducer exclusion, inducer expulsion and inducer control is described. EI, Enzyme I; HPr, heat-stable phosphocarrier protein; Glc-6P, glucose-6P; Pase II, sugar phosphatase II; CcpA, catabolite control protein A; RNA polym., RNA polymerase; H^+, proton; S, secondary transport protein; CRE, catabolite responsive element; PEP, phosphoenolpyruvate; FDP, fructose-1,6-disphosphate; and P_i, inorganic phosphate. Adapted from Poolman (2002).

1.8.5. Transport of sugars

1.8.5.1. Glucose transport

The major transport system of glucose in *L. lactis* is a mannose-PTS system (PTS^{man}), which is quite unspecific and also transports 2-deoxy-D-glucose, mannose, glucosamine and fructose (Neves et al. 2005). Glucose is transported and concomitantly phosporylated to glucose 6-phosphate by EIIA. Another glucose-PTS system possessing specificity to glucose and α-methyl-glucoside has also been described for some strains (Neves et al. 2005). Glucose can alternatively be transported by a secondary transport system using sugar permeases, especially in heterofermentative LAB such as *Lactobacillus reuteri* (Taranto et al. 1999).

1.8.5.2. Fructose and sucrose transport

L. lactis uses either the same PTS^{man} as glucose in fructose transport, yielding fructose 6-phosphate, or a fructose-PTS resulting in fructose 1-phosphate. Fructose 1-phosphate is then phosphorylated to FDP before it enters glycolysis. Some heterofermentative LAB, e.g. *L. reuteri* and *Lactobacillus brevis*, also use PTS for fructose transport (Saier et al. 1996, Taranto et al. 1999). Sucrose is a disaccharide that consists of glucose and fructose units. Sucrose is transported by sucrose-specific PTS, resulting in sucrose 6-phosphate. In turn, sucrose 6-phosphate is hydrolyzed by sucrose 6-phosphate hydrolase, yielding glucose 6-phosphate and fructose. Glucose 6-phosphate enters glycolysis as such, whereas the fructose unit must first be phosphorylated by an ATP-dependent fructokinase (Neves et al. 2005).

1.9. Control of sugar metabolism

The transporters are also a part of the regulatory mechanisms in the sugar metabolism of LAB. The LAB optimize and tune their carbohydrate utilization using two regulatory mechanisms, hierarchical control and autoregulation control. The autoregulation system controls the catabolic activities within a specific metabolic pathway, whereas the hierarchical control acts as the selector of preferential catabolic pathways (Poolman 2002).

The phosphotransferase system (PTS) has an essential role in both control systems of carbohydrates by using the HPr protein as the central regulatory protein. The HPr can be phosphorylated at the histidine-15 residue in the PTS cycle resulting in HPr(His-P) or phosphorylated at the serine-46 by the HPr kinase /phosphatase (HPrK/P), resulting in HPr(Ser-P). When cells are in a high catabolic state, high intracellular levels of ATP and/or FDP stimulate HPr kinase/phosphatase (HPrK/P) activity, which increases the HPr(Ser-P) level. Because HPr(Ser-P) is not a substrate for the EI protein, the PTS cycle is hindered and sugar uptake and catabolism are reduced (Axelsson 2004, Titgemeyer & Hillen 2002). The stimulation and inhibition of HPrK/P by different intracellular metabolites seems to be species specific.

1.9.1. Hierarchical control

Hierarchical controls are composed of (1) CcpA-mediated catabolite repression, where inhibition of the expression of genes encoding enzymes is involved in the transport and metabolism of less preferred carbohydrates, (2) inducer exclusion, including inhibition of the activity of enzymes that affect the uptake or production of a transcriptional inducer, and (3) inducer expulsion, including stimulation of the efflux of an intracellular inducer, that is, a carbohydrate or its phosphorylated derivative (Poolman 2002). The latter two control systems also affect gene expression, but indirectly by lowering the concentration of the intracellular inducer (Figure 6).

1.9.1.1. CcpA-mediated catabolite repression

The CcpA is a general transcription factor that has a central role in the repression of several catabolic genes (Poolman 2002). Hpr(Ser-P) interacts with CcpA protein, forming a complex where CcpA binds to a *cis*-acting sequence termed the catabolite responsive element (*cre*) and represses transcription (Figure 6) (Titgemeyer & Hillen 2002). The *cre* sequence is located in or close to the promoter region of a catabolic operon (Poolman 2002).

CcpA-mediated catabolite repression is interlinked with PTS. The uptake of glucose or other rapidly metabolizable PTS sugars and the concomitant dephosphorylation of PTS protein increases the concentrations of glycolytic intermediates that activate HPr kinase/phosphatase (HPrK/P). The consequence of this is an elevated Hpr(Ser-P) level and thus the repression of CcpA dependent genes (Poolman 2002). The *ccpA* gene itself is probably autoregulated by using the *cre* site in the *ccpA* promoter region (Titgemeyer & Hillen 2002). The CcpA can also act as a transcriptional activator, like in the lactic acid synthesis (*las*) operon of *L. lactis*. The *las* operon controls three glycolytic enzymes: phophofructokinase, pyruvate kinase and lactate dehydrogenase (Poolman 2002).

1.9.1.2. Inducer exclusion

Inducer exclusion involves the allosteric inhibition of transporter proteins by Hpr(Ser-P) or HPr(His-P), which prevents the transport of the inducer sugars into the cell (Figure 6) (Poolman 2002, Titgemeyer & Hillen 2002). In addition, the HPr(His-P) affinity differs for the sugar-specific IIA proteins/domains, which leads to competition for HPr(His-P) and the hierarchical utilization of PTS sugars (Poolman 2002).

1.9.1.3. Inducer expulsion

In inducer expulsion, the inducer that causes catobolite repression is expelled from the cell (Figure 6). In homofermentative LAB the addition of rapidly metabolizable sugar results in dephosphorylation of accumulated sugar-P by a sugar-P phosphatase (PaseII), and hence this formed free sugar is excreted from the cell. HPr(Ser-P) probably stimulates the PaseII (Poolman 2002).

In heterofermentative LAB, it is considered that binding of HPr(Ser-P) to glucose/H^+ and lactose/H^+ symporters alters the energy coupling mechanism, resulting in the conversion of carbohydrate-proton symport into carbohydrate uniport. As a consequence, the accumulated free sugars, lactose and glucose, are expelled from LAB cells down their concentration gradients (Poolman 2002).

1.9.2. Autoregulatory control

Autoregulatory control of carbohydrate utilization adjusts the rate of transport of a particular carbohydrate according to the rate of its metabolism and the availability of the substrate, thereby providing feedback or feedforward control to the pathway. Autoregulation involves similar control mechanisms as in the hierarchical control. These are CcpA mediated control of transcription by HPr(Ser-P) and the control of non PTS enzyme/transport activity by HPr(His-P) mediated phosphorylation (Figure 6). The HPr(Ser-P)/HPr(His-P) ratio changes in the cells during different growth stages and is controlled by intracellular levels of metabolic substrates (FDP, P_i, PEP and ATP) (Poolman 2002).

2. GENETIC TOOLS IN THE METABOLIC ENGINEERING OF LAB

Different genetic tools are needed in the metabolic engineering of bacteria. These genetic tools are used to delete, remove, combine and replace the genes, as well as to express/overexpress heterologous or homologous genes in the host cell. Traditionally, the LAB strains have been improved for different industrial applications by using random mutagenesis, after which the desired phenotype is screened (Smid et al. 2005b). Several efficient genetic tools have been developed to allow more specific engineering of the metabolism of the LAB during the last couple of decades.

2.1. Random mutagenesis

Mutation is a permanent alteration of a nucleotide or nucleotides at a specific site in the DNA strand. The mutation could be a point mutation (change of a single nucleotide), deletion, substitution or rearrangement of DNA. Traditional random strain mutagenesis is still very practical when adequate molecular information is lacking (Bai et al. 2004, Parekh et al. 2000). Random mutagenesis is a quite simple and rapid method when compared to targeted mutagenesis using recombinant DNA techniques, but the selection/screening of the desired mutant strain is often challenging in random mutagenesis.

The LAB are generally mutagenized by chemicals such as the strong alkylating agents methyl methanesulfonate (MMS) or N-methyl-N-nitro-N´-nitrosoguanidine (MNNG) (von Wright & Sibakov 1998) or by ultraviolet radiation (Bai et al. 2004). Transposons are also used in random mutagenesis. In this system, the random mutagenesis is derived from the replicative transposition of the IS element in a plasmid vector and integration of this plasmid/IS transposon complex into the chromosome (Mills 2001).

2.2. Gene transfer systems

Genetic modification requires a delivery system for the transfer of genetic material into the bacterial cell. Gene transfer can occur by natural mechanisms such as *in vivo* physiological transformation, transduction or conjugation. *In vitro* gene transfer systems such as electroporation and protoplast transformation have been developed to obtain more efficient transformation systems for a wide variety of LAB strains (Morelli et al. 2004).

2.2.1. Electroporation

Electroporation is the most common method to transform plasmid vectors into LAB cells (Mills 2001). In electroporation, the bacterial cells are grown, washed and suspended in the electroporation buffer. The cell wall is rendered permeable to DNA (plasmid vector) by using an electric impulse conducted through the cell suspension. After the electric pulse, the cell suspension is diluted with the incubation medium and incubated to recover the cells before plating on a selective medium. Electroporation methods have been optimized for several LAB species and genera (e.g. *Enterococcus*, *Lactobacillus*, *Lactococcus* and *Leuconostoc*). The efficiency of electroporation varies substantially between different LAB strains and their transformation frequency can be from 10^1 to 10^7 transformants per microgram of DNA (Berthier et al. 1996, Luchansky et al. 1988, Serror et al. 2002, Wei et al. 1995).

2.2.2. Optimization of electroporation

The transformation efficiency of electroporation is strain dependent and therefore several parameters have to be tested to optimize the electroporation for each new LAB strain. The parameters to be optimized for the electoporation are: the growth stage and growth medium, the composition of the wash and electroporation buffers, electrical pulse parameters, and the source and amount of plasmid DNA.

Gram-positive bacteria have a thick cell wall, which lowers the efficiency of the transformation (Kim et al. 2005). Therefore, a number of treatments have been used to make the cell wall more permeable: (1) the addition of glycine, threonine or penicillin to the growth medium, (2) treatment of the cell wall with lysozyme, and (3) thermal treatment to induce autolysis (Berthier et al. 1996, Kim et al. 2005, Wei et al. 1995). In general, the addition of 0.1–1% glycine to the growth medium has been found to improve the transformation frequency of electroporation in many LAB (Bhowmik & Steele 1993, Serror et al. 2002, Wei et al. 1995). However, a concentration of glycine as high as 8% has even been used with *L. plantarum* (Thomson & Collins 1996). Also, the growth stage affects the rigidity and thickness of the cell wall, and bacterial cells are therefore usually harvested from the exponential phase to the early stationary phase for electroporation.

2.2.2.1. Washing/electroporation buffers and incubation medium

Washing/electroporation buffers are used to wash and protect bacterial cells during electroporation. Washing/electroporation buffers commonly have glycerol and/or sucrose as an osmostabilizer. The addition of $MgCl_2$ to the washing buffer helps the removal of extracellular polysaccharides, which seem to have a negative effect on the transformation (Berthier et al. 1996). Supplemented $MgCl_2$ also has a role in the stability of the cytoplasmic membrane. In addition, the presence of Mg^{2+} or other divalent cations in the incubation medium before plating may increase the transformation efficiency by allowing better maintenance of the plasmids in the recipient cell (Berthier et al. 1996).

2.2.2.2. Electrical parameters

The electrical parameters, namely the voltage, resistance and electrical pulse duration affect the permeabilization of the cell, which allows the delivery of DNA into the cell. The used buffers, bacterial strain, the quantity of cells and the amount of DNA affect the selectable electrical parameters. For example, salt/ions in the electroporation buffer or in the DNA sample increase the electrical conductivity, which may cause electrical overload if the selected values of the electrical parameters are too high. The dilemma in optimizing the electrical parameters is to obtain the highest transformation efficiency but at the same time to minimize the damage to the cell (Kim et al. 2005). According to Berthier et al. (1996), the optimum transformation efficiency for *Lactobacillus sakei* was obtained when either a high voltage/low resistance (short pulses) or low voltage/high resistance (long pulses) was used. The same general pattern also seems to work with many other lactic acid bacteria.

2.3. Cloning Vectors

The cloning vectors are constructed from plasmids, which are extrachromosomal, autonomous and self-replicating DNA elements present in prokaryotes and some eukaryotes. Many plasmids found in LAB are cryptic, meaning that they have no apparent function (Shareck 2004). Many constructed cloning vectors are based on small cryptic plasmids or large conjugative plasmids of different LAB strains (Pérez-Arellano et al. 2001, von Wright & Sibakov 1998). A typical cloning vector consists of an origin of replication (*ori*), a selectable marker gene to identify the transformed cells (usually an antibiotic marker) and a set of restriction endonuclease sites for insertion of the DNA fragment. A small size is also a desired property because it increases the structural and segregational stability of the vector (Shareck 2004).

The cloning vectors of the LAB and other gram-positive bacteria are divided into two major classes. The class 1 vectors are the large conjugative plasmids like pIP501 (30.2 kb) and pAMβ1 (26.5 kb), which replicate by theta mechanisms (Shareck 2004, von Wright & Sibakov 1998). These plasmids and their derivatives have segregational and structural stability and they can replicate in various LAB such as *Lactococcus* spp., *Lactobacillus* spp. and *Pediococcus* spp. and many other gram-positive bacteria (Shareck 2004).

The class 2 vectors are based on cryptic lactococcal plasmids like pSH71 (2.1 kb) and pWV01 (2.2 kb). These plasmids usually use rolling circle mechanisms to replicate. The rolling circle replication mechanism is likely to cause more segregational and structural instability in the plasmids than the theta mechanisms, and there may therefore be problems in maintaining large DNA fragments (Shareck 2004, von Wright & Sibakov 1998). However, the ability of SH71 and pWV01 replicons and their derivates to replicate in *E. coli* and many gram-positive bacteria make them useful for cloning purposes (Shareck 2004). The prototype cloning vectors from SH71 and pWV01 replicons are pNZ12 (pNZ series) and pGK12 (pGK-series), respectively.

2.3.1. Integration vectors

Integration vectors have been used for gene knock-out, amplification, replacement and insertion. Three types of integration system have been used: (1) transposition via IS-elements, (2) site-spesific recombination using att/integrase systems, and (3) homologous recombination via suicide or temperature sensitive vectors (Shareck 2004).

2.3.1.1. Homologous recombination

In homologous recombination the integration vector carries a homologous DNA fragment, which facilitates integration into the chromosomal target DNA sequence when the replication of the vector is hindered in the host cell. The recombination event is named single crossover or Campbell-like integration when the entire plasmid with the homologous sequence is integrated into a defined genetic locus. In this way the target gene(s) can be inactivated or inserted into a specific location. The single crossover recombination is a reversible event, but the integration can be maintained by applying selective pressure to the integration plasmid. In double crossover, a second recombination occurs between the homology regions of the integrated single crossover vector and the chromosomal DNA, resulting in the excision of the vector from the chromosome (Shareck 2004). Double crossover results in either the replacement of the host chromosomal locus by the new DNA fragment in the chromosome or restoration of the original DNA fragment (Leenhouts et al. 1996).

2.3.1.2. Temperature-sensitive plasmids

Chromosomal integration is based on non- or conditionally-replicating plasmids. The pG^+ series of integration vectors are conditionally-replicating plasmids that have a temperature-sensitive replicon. These temperature-sensitive vectors can be transformed and maintained in gram-positive and gram-negative hosts at a permissive temperature (e.g. 28°C). However, if the growth temperature is elevated (e.g. above 35°C), the replication initiation protein (RepA) is inactivated. The inactivation of RepA hinders the replication of the vector and forces it to integrate into the host chromosome with the concomitant selection pressure.

Other gene replacement systems have also been devised using the temperature-sensitive replicon. One approach is to use a $RepA^+$ temperature sensitive helper plasmid, pVE6007 (Maguin et al. 1992), and a $RepA^-$ vector, pORI280 (Leenhouts et al. 1996), which

delivers a homologous DNA fragment. Both plasmids are maintained in a permissive temperature, but when the growth temperature is elevated the RepA is inactivated in the pVE6007 helper plasmid. Without the RepA the pORI280 is unable to replicate and it is integrated into the chromosome (Shareck 2004).

Russell and Klaenhammer (2001) have developed a temperature-sensitive helper plasmid, pTRK669, based on the pWV01 replicon for thermophilic lactobacilli. In addition, Neu and Henrich (2003) have developed an integration vector for thermophilic lactobacilli based on the pTN1 vector. This plasmid was derived from the tpLC2 rolling-circle plasmid of *L. curvatus* and its replication is efficiently shut down at 42°C (Neu & Henrich 2003).

2.3.2. Expression vectors

Expression vectors are needed to express homologous or heterologous genes in the LAB host. These vectors include suitable promoters to control the transcription (and translation) of the cloned gene. Often, the transcription terminator is attached downstream of the cloned gene to end the gene transcription and to avoid unnecessary transcription of the vector. Signal sequences can be added to allow the export of the produced protein. In addition, to facilitate the isolation of the produced protein, the His-tag or some other affinity tag can be attached.

If the promoter is strong and the expression of the gene product is too high, the protein may accumulate in the cytoplasm and produce biologically-inactive inclusion bodies. Also, some gene products are toxic to the cell when expressed at a moderate/high concentration (Morelli et al. 2004). Therefore, several inducible promoters for the control of the expression level have been developed. These include sugar- and NaCl-regulated promoters, a pH decrease, a temperature upshift and phage infection promoters (Shareck 2004). So far, only the nisin inducible promoter system, named NICE, has found widespread use in LAB, particularly in *L. lactis* (Morelli et al. 2004).

2.3.2.1. NICE system

Nisin is an antimicrobial peptide, and its biosynthesis is controlled by a nisin gene cluster. The NICE system is based on signal transduction by a two-component regulatory system, consisting of the sensor histidine kinase, NisK, and the response regulator NisR, found in the nisin gene cluster of *L. lactis* (Kuipers et al. 1997). The NICE system includes a plasmid that has the desired gene under the control of the *nisA* promoter fragment. This plasmid is introduced in a strain that cannot produce nisin but is able to produce the NisR and NisK proteins. Addition of nisin to growth medium during logarithmic growth results in effcient transcription of the gene under the control of the *nisA* promoter (Kuipers et al. 1997). The above-described expression systems using the integrated *nisR* and *nisK* host strains are the most common among *L. lactis* strains. *nisR* and *nisK* integrated strains have also been developed for thermophilic *Lactobacillus gasseri* (Neu and Henrich 2003). The expression level is proportional to the amount of

nisin added and the level of intracellular production of different proteins may range up to 10% to 60% of the total soluble protein (Kuipers et al. 1995, Kuipers et al. 1997).

In addition to *L. lactis* host, with integrated *nisR* and *nisK* genes, other versions of *L. lactis* (and other LAB) strains and plasmids have also been developed for the nisin inducible expression system (de Ruyter et al. 1996). These include a two-vector system with one of the plasmids containing the expressed gene fused with the *nisA* promoter and the other plasmid (broad-host-range) containing the *nis*R and *nis*K genes. This nisin-controlled system also allows expression in several other gram-positive bacteria (Morelli et al. 2004), although the nisin-controlled expression range is often lower than in *L. lactis*. Another induction system based on a single vector, which contains the *nisA* and *nis*R-*nis*K together, as well as an *E. coli* and a gram-positive replication origin, has been described by Bryan (2000).

A problem associated with the nisin induction system, particularly in LAB other than *L. lactis*, is to find a correct expression level for *nis*R and *nis*K that can be tolerated by the host cell and still allow efficient induction. However, the nisin control expression system has many advantages for protein production applications. Nisin has food-grade status, it is low-cost and only small amounts (0.05 - 5 ng/ml) are needed in induction. In addition, the nisin system is easy to use and its expression is tightly controlled, depending on the amount of nisin added (Kuipers et al. 1997).

2.3.2.2. Synthetic promoters

To optimize the expression level and to obtain a more stable expression system for the targeted gene(s), Jensen and Hammer (1998a) have developed a system based on a library of synthetic promoters for *L. lactis*. The library consists of promoters that differ in strength over 3 to 4 logs of activity. The -35 and -10 consensus sequences of these promoters were kept constant while the spacer sequences between them were randomized. The promoter fragments, including the consensus sequences and the random spacer sequences, were synthesized by using the degenerated single-stranded promoter oligonucleotide. This designed oligonugleotide was converted to double-stranded DNA by using DNA polymerase and an oligonugleotide primer complementary to the 3´end of the degenerated primer (Jensen & Hammer 1998a).

The mixture of promoter fragments was cloned into the vector carrying the β-galactosidase genes (*lacL* and *lacM*). The different randomized spacer sequences of the promoters allowed the expression of the β-galactosidase genes over a wide range of activity by small steps (Jensen & Hammer 1998a, b). Solem and Jensen (2002) have modified the method to construct the promoter library by connecting the synthetic promoters directly to the target gene(s). This method lessens and simplifies the cloning work to obtain an optimal expression level of the target gene(s) (Solem & Jensen 2002). The method could be widely applied in different LAB (as well as other bacteria) provided that a suitable screening system for the target gene expression is available.

3. ENGINEERING OF METABOLIC PATHWAYS IN LAB

LAB are promising targets for metabolic engineering, because their energy and carbon metabolism is relatively simple and energy metabolism is not generally connected to biosynthetic activity. Therefore, the sugar metabolism pathways can be manipulated without disturbing the synthesis of cell components (Kleerebezem & Hugenholtz 2003). Many metabolic engineering strategies for LAB have focused on rerouting pyruvate metabolism to produce commercially important end products (sweeteners, flavors and aroma components) (Smid et al. 2005a). Metabolic engineering of more complex biosynthetic pathways leading, for example, to exopolysaccharides, vitamin B2 and B11 has been reported (Kleerebezem & Hugenholtz 2003, Sybesma et al. 2004). Being one of the model organisms in microbial metabolism, *L. lactis* has obviously also been the main target of metabolic engineering in LAB.

3.1. Lactic acid

Lactic acid (or lactate) is used in food as a preservative and flavor enhancer, for moisturizing and emulsifying in cosmetics, and in the synthesis of optically pure pharmaceuticals (van Maris 2004). Lactic acid has two optical stereoisomers, the (D-) and L (+) isomers. L-lactate is preferred for food and pharmaceutical applications because it is a normal intermediate in mammalian metabolism. However, D-lactate has been considered to be a non-physiological isomer, which could cause adverse effects for infants and patients suffering from short-bowel syndrome and intestinal failure (Lapierre et al. 1999). L-lactate is also used industrially as the starting material in the production of, for example, valuable synthetic biopolymers (Bai et al. 2004). In chemical synthesis, a racemic mixture of these two stereoisomers of lactic acid, D (-) and L (+), is produced. However, in lactic acid fermentation it is possible to synthesize either the D- or L-lactic acid, or both. LAB use two isomer-specific enzymes, D- and L-lactate dehydrogenases, to produce the respective lactic acid isomers. In addition, in some strains (e.g. *L. plantarum* and *L. sakei*) there is a racemase activity that is responsible for the conversion of L-lactate into D-lactate (Goffin et al. 2005)

3.1.1. Production of lactic acid by metabolic engineering

Many LAB are naturally quite optimal lactic acid producers. Hence, most of the improvements of LAB for producing more lactic acid have focused on optimization of the fermentation process and the selection/screening of good lactic acid producers. Therefore, metabolic engineering studies have focused on the production of pure L-lactic acid in homofermentative LAB. For instance, Bhowmik and Steele (1994) carried out insertional inactivation of a D-lactate dehydrogenase gene (*ldhD*) from *Lactobacillus helveticus*, resulting in the production of pure L-lactate. However, the total amount of produced lactic acid was the same as in wild type *L. helveticus*, although the *ldhL* gene was expressed in a high copy number plasmid. Kylä-Nikkilä et al. (2000) also studied lactic acid production in *L. helveticus* and constructed two stable *ldhD*-negative strains by using chromosomal integrations. These *L. helveticus* strains produced only L-lactic acid. In the first strain the *ldhD* gene promoter region was deleted, while in the other strain

ldhD was replaced by an additional copy of *ldhL*. The additional *ldhL* under the control of the *ldhD* promoter was meant to enhance the synthesis of L-lactic acid in the stationary phase. The overall results were similar to those of Bhowmik and Steele (1994). However, at a low pH the double *ldhL* strain produced 20% more L-lactic acid than the strain carrying only the deleted *ldhD* promoter (Kylä-Nikkilä et al. 2000).

Inactivation of *ldhD* in *Lactobacillus johnsonii* also resulted in the production of pure L-lactate, but some pyruvate was also converted to other end products (e.g. diacetyl and acetoin) (Lapierre et al. 1999). Inactivation of the *Lactobacillus plantarum ldhD* gene instead resulted in the production of a racemic mixture of the lactate (Ferain et al. 1996), while inactivation of the *ldhL* gene resulted exclusively in the production of D-lactate (Ferain et al. 1994). As in *L. helveticus*, overexpression of *ldhL* in *L. plantarum* hardly had any effect on the production of L- and D-lactate (Ferain et al. 1994). Furthermore, increasing the copy number of the *las* operon, including the *ldhL*, phosphofructokinase (*pfk*) and pyruvate kinase (*pyk*) genes, in *Lactoccus lactis* resulted in only a small increase in lactic acid production (Davidson et al. 1995). Recently, Bai et al. (2005) carried out UV mutagenization of a *Lactobacillus lactis* strain and produced an L-lactate overproducing strain. The mutagenization was proposed to affect the elimination of the high level of NADH oxidase activity and increase the uptake rate of glucose, thus enhancing L-lactate production (Bai et al. 2005).

3.2. Production of diacetyl

Diacetyl provides the typical butter aroma in dairy products. It is produced from α-acetolactate through oxidative decarboxylation (see chapter on the diacaetyl/acetoin pathway). Especially *L. lactis* subsp. *lactis* biovar. *diacetylactis* produces diacetyl from citrate in cometabolic fermentation with lactose (Kleerebezem et al. 2000). Citrate is, however, a minor compound in dairy products and hence different strategies to increase diacetyl production from lactose instead of citrate have been one of the main targets in the metabolic engineering of *L. lactis* (Hugenholtz et al. 2000, Kleerebezem et al. 2000).

One approach to increase diacetyl production in *L. lactis* has been to disrupt its *ldh* gene. The resulting mutant strain produced high amounts of formate and ethanol in anaerobic conditions, but under aerobic conditions acetate and acetoin were formed from pyruvate as the main end products (Kleerebezem et al. 2000, Platteeuw et al. 1995). Another approach has been to increase the amount of α-acetolactate synthase (ALS) or acetohydroxy acid synthase enzymes (ILVBN), which convert pyruvate to α-acetolactate (Kleerebezem et al. 2000). However, the overexpression of these enzymes in *L. lactis* combined with growth under aerobic conditions only increased the flux from the pyruvate pool towards acetoin (Kleerebezem et al. 2000, Platteeuw et al. 1995).

The combination of these two different approaches, in which ALS had been overexpressed in an LDH-deficient mutant strain, merely led to the production of low amounts of α-acetolactate and high amounts of acetoin (Kleerebezem et al. 2000). Inactivation of α-acetolactate decarboxylase (ALDB), an enzyme that transforms α-acetolactate to acetoin, also resulted in an *L. lactis* strain that produced only low amounts

of diacetyl under aerobic conditions. Slightly more diacetyl was produced in this ALDB-deficient strain with the overexpressed ILVBN (Kleerebezem et al. 2000, Swindell et al. 1996). An *L. lactis* strain that would have all these three mutations together (ALS or ILVBN overproduction as well as LDH- and ALDB-deficiency) might be an effective diacetyl producer, but so far the construction of such a strain has not been reported. The reason for this might be the distortion of the redox balance in lactococcal cells, which could lead to poor viability (de Vos & Hugenholtz 2004, Kleerebezem et al. 2000).

3.2.1. Diacetyl production by cofactor engineering

The intracellular redox balance (NAD^+/NADH) has a predominant role in the control of different fermentation patterns (Hols et al. 1999b, Kleerebezem et al. 2002, Lopez de Felipe et al. 1998). The redox balance in an *L. lactis* strain was regulated by using the *Streptococcus mutans nox* gene, which was under the expression control of the NICE system (Lopez de Felipe et al. 1998). NOX overproduction in the presence of oxygen resulted in large reduction in the intracellular pool of NADH ($1/2\ O_2 + H^+ + NADH$ à $H_2O + NAD^+$), thus rerouting the pyruvate pool towards oxidative or NADH-independent pathways in *L. lactis* (Hugenholtz et al. 2000, Kleerebezem et al. 2000). By overproducing NOX in an ALDB-deficient *L.lactis* strain and using these cells in a nongrowing state under aerobic incubation, about 80% of the pyruvate pool was successfully rerouted towards α-acetolactate and diacetyl (Hugenholtz et al. 2000).

3.3. L-alanine production

L-alanine is used as a food sweetener and for pharmaceutical applications (Hols et al. 1999a). L-alanine dehydrogenase (L-AlaDH) from *Bacillus sphaericus* was overexpressed by using the NICE system in *L. lactis* to produce L-alanine. Both L-AlaDH and L-LDH use pyruvate as the substrate and NADH as the cofactor. The K_m values of the *B. sphaericus* L-AlaDH for pyruvate and NADH are also similar to the corresponding K_m values of *L. lactis* L-LDH (Hols et al. 1999a). Glucose fermentation in the presence of excess ammonium with *L. lactis* overexpressing the *B. sphaericus* L-AlaDH resulted in the production of alanine at the level of 35% of the end products. Futhermore, an L-LDH deficient strain of *L. lactis* with L-AlaDH overexpression completely converted glucose into alanine. However, the produced alanine was a mixture of D- and L-alanine stereoisomers, where L-alanine was converted to D-alanine by alanine racemase. Thus, to obtain only stereo-specific L-alanine the gene endcoding alanine racemase (ALR) was distrupted in the *L. lactis* L-LDH deficient strain. Finally, using this ALR/L-LDH deficient mutant in the resting cell fermentation, the exclusive production of L-alanine was achieved (Hols et al. 1999a).

3.4. Production of acetaldehyde

Acetaldehyde is also an important aroma compound in dairy products, especially in yoghurt. Two LAB starter strains, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, were found to produce acetaldehyde in yoghurt (Bongers et

al. 2005). LAB have several metabolic pathways for acetaldehyde formation, including amino acids, nucleotide and pyruvate metabolism (Chaves et al. 2002).

Some attempts have been made to improve acetaldehyde production in LAB. In *S. thermophilus*, the overexpression of endogenous serine hydroxymethyltransferase (SHMT), encoded by the *glyA* gene, has resulted in overproduction of acetaldehyde. The SHMT enzyme possesses threonine aldolase activity, which converts threonine into acetaldehyde and glycine. The increase in acetaldehyde production of *glyA*-overexpressing strains was about 80–90% (Chaves et al. 2002). Expression of pyruvate decarboxylase (*pdc*) from the gram-negative bacteria *Zymomonas mobilis* in *L. lactis* rerouted pyruvate towards acetaldehyde. The overexpressed pyruvate decarboxylase, controlled by the NICE system, competes with lactate dehydrogenase for the available pyruvate. To further increase the pyruvate availability for acetaldehyde production, the NADH oxidase (*nox*) gene was also overexpressed. This NOX and pyruvate decarboxylase (PDC) overproduction mutant converted almost 50% of the glucose consumed to acetaldehyde under anaerobic conditions (Bongers et al. 2005).

4. THE PRODUCTION OF SOME OTHER INDUSTRIALLY IMPORTANT COMPOUNDS

4.1. Mannitol

D-Mannitol is a six-carbon acyclic sugar alcohol that is synthesised by a diverse group of organisms such as bacteria, fungi, algae, lichens and higher plants (Bär 1985, Ikawa et al. 1972, Loescher 1987, Rumpho et al. 1983). It is a low-calorie sugar alcohol that can replace sucrose, lactose, fructose or glucose in food products. Mannitol can also serve as an antioxidant in biological cells (Hugenholtz et al. 2002). In the human intestine, mannitol may be converted into short-chain fatty acids, which have been claimed to prevent the development of colon cancer. On the other hand, mannitol can result in gastrointestinal discomfort, such as flatulence and diarrhea, because it is slowly and only partially absorbed in the small intestine (Neves et al. 2005)

4.1.1. Production of mannitol

Commercially, mannitol is produced by catalytic hydrogenation of glucose-fructose syrup or inverted sugar with the co-production of sorbitol (Soetart et al. 1999). In these processes, mannitol is a minor by-product, being also relatively difficult to separate from sorbitol. Yeast, fungi and lactic acid bacteria (LAB) could synthesise mannitol without co-formation of sorbitol (von Weymarn et al. 2002). Heterofermentative LAB are known to convert fructose to mannitol by using mannitol dehydrogenase (MDH). In this reaction, part of the fructose is directed to the heterofermentative pathway while the other part acts as an electron acceptor and is reduced to mannitol (see chapter 1.5). Mannitol production is increased in this reaction if fructose is co-fermented with glucose (Hugenholtz et al. 2002). High mannitol yields have been achieved by optimizing the mannitol fermentation of heterofermentative LAB. Especially *L. mesenteroides* was

found to have a high volumetric productivity (26.2 g l^{-1} h^{-1}) with a 97 mol% mannitol yield from fructose in resting state fermentation (von Weymarn et al. 2002).

The homofermentative LAB have also been reported to produce mannitol in unusual conditions. Disruption of lactate dehydrogenases (LDH) in *L. plantarum* and *L. lactis* resulted in the production of mannitol and other end products (Ferain et al. 1996, Hugenholtz et al. 2002, Neves et al. 2000, 2005). In *L. lactis*, the disruption of *ldh* induced an increase in the levels of mannitol 1-phosphate dehydrogenase (MPDH) and mannitol 1-phosphate phosphatase (MP), enzymes that are both involved in the synthesis of mannitol from fructose-6-P (Neves et al. 2005). In the synthesis of mannitol, the MPDH reduces fructose-6-P to mannitol-1-P, which is subsequently dephosphorylated to mannitol by MP (Hugenholtz et al. 2002).

The yield of mannitol production in homofermentative LAB has been low as compared to mannitol production of heterofermentative LAB. However, Gaspar et al. (2004) succeeded in constructing higher-yielding *L. lactis* strains. The mannitol transport system of the LDH-deficient *L. lactis* strain was inactivated by deleting the *mtlA* or *mtlF* genes encoding $EIICB^{Mtl}$ and $EIIA^{Mtl}$, respectively. In the resting state, these double mutant strains converted about 30% of glucose to mannitol (Gaspar et al. 2004). Moreover, Wisselink et al. (2004) constructed an LDH-deficient *L. lactis* strain that overexpressed the mannitol 1-phosphate dehydrogenase gene (*mtlD*) derived from *L. plantarum*. Using nisin-inducible expression, this strain converted 25% of glucose to mannitol in resting state fermentation. Wisselink et al. (2005) achieved the highest conversion yield from glucose to mannitol (50%) with an *L. lactis* LDH-deficient strain that overexpressed the mannitol 1-phosphate dehydrogenase gene (*mtlD*) of *L. plantarum* together with a mannitol 1-phosphate phosphatase of *Eimeria tenella* (a protozoan parasite). This 50% yield was obtained by using growing cells and is close to the theoretical mannitol yield of 67% in *L. lactis* (Wisselink et al. 2005). (Production of mannitol in LAB is further discussed in Studies I and III).

4.2. Sorbitol

Sorbitol, like mannitol, belongs to the sugar alcohols and naturally occurs in many fruits. Sorbitol has applications in the food industry as a sweetener, a humectant and a texturizer. It is also used in different pharmaceutical products and the synthesis of vitamin C (Silveira & Jonas 2002). Sorbitol is traditionally produced by catalytic hydrogenation of glucose (Silveira & Jonas 2002, Nyyssölä & Leisola 2005). As described in the preceding section on mannitol, sorbitol is one of the main products during the co-production of mannitol in the catalytic hydrogenation of glucose-fructose syrup (Soetart et al. 1999). Only a few organisms have been described as able to produce sorbitol. *Candida boidinii*, *Candida famata* and *Saccharomyces cerevisiae* are able to produce sorbitol, but no further studies on the biotechnological production of sorbitol with these yeasts have been reported. However, the gram-negative bacterium *Zymomonas mobilis* offers potential for the biotechnological production of sorbitol and hence many attempts have been made to develop a *Z. mobilis* based production system for an effective industrial process (Silveira & Jonas 2002).

In LAB, the metabolicengineering of facultative heterofermentative *L. plantarum* and *L. casei* for sorbitol production has been reported. Mannitol phosphate dehydrogenase (MPDH) and lactate dehydrogenase (LDH) were inactivated in an *L. plantarum* strain overexpressing a sorbitol dehydrogenase gene (*stlDH*). This resulted in considerable sorbitol production. Both MPDH and sorbitol dehydrogenase (SDH) use fructose-6-P as a substrate; thus the inactivation of the MPDH allowed more fructose-6-P to be reduced to sorbitol-6-P by sorbitol dehydrogenase (Hugenholtz et al. 2002).

Nissen et al. (2005) constructed an *L. casei* strain in which the sorbitol-6-phosphate dehydrogenase gene (*gutF*) was integrated into the chromosomal lactose operon (*lac*). Consequently, this gene was under the control elements of the *lac* operon, which is repressed by glucose and induced by lactose. Using these recombinant resting cells, which were pre-grown on lactose, the cells were able to synthesize sorbitol from glucose. In addition, the inactivation of LDH from this *L. casei* recombinant strain apparently resulted in a higher NADH pool in the cells, which increased the conversion rate of glucose to sorbitol from 2.4% to 4.3% (Nissen et al. 2005). However, these production levels are quite far from that of *Z. mobilis*, which can convert 91-100% of fructose to sorbitol (Silveira & Jonas 2002).

4.3 Xylitol

Xylitol is a five-carbon sugar alcohol that is found in small amounts in nature, mainly in plants, vegetables and fruits. Xylitol is used as a sweetener in the food industry and it has a similar sweetening capacity to sucrose. Xylitol is also used in dental products because of its anti-cariogenic properties (Nyyssölä & Leisola 2005). At present, xylitol is produced through the chemical reduction of xylose, using a nickel catalyst (Nyyssölä & Leisola 2005). The chemical process is expensive because hydrogenation of D-xylose requires a high temperature and pressure, and because of the high purity demand for D-xylose (Rao et al. 2005).

Microbes such as yeasts, fungi and bacteria are able to reduce D-xylose to xylitol by xylose reductase. Therefore, the biotechnical production of xylitol could be attractive, especially if the industrial side-streams could be used as substrates instead of pure D-xylose. Currently, yeasts belonging to the genus *Candida* have been the most efficient xylitol producers (Nyyssölä & Leisola 2005).

4.3.1 Production of xylitol in LAB

Natural LAB strains are not reported to produce xylitol, although some strains of *Streptococcus avium* and *Lactobacillus casei* are able to metabolize it (London 1990, Nyyssölä & Leisola 2005). However, Nyyssölä et al. (2005) have studied a novel recombinant *L. lactis* strain as a production host that is able to produce xylitol using a glucose-xylose mixture as the sugar substrate. This *L. lactis* strain overexpressed the xylose reductase from the yeast *Pichia stipitis* and a xylose transporter from *L. brevis*. The aim of expression of the xylose transporter was to enhance the influx of xylose to the cell. However, the expression of the xylose transporter with co-expression of the xylose

reductase had only a small beneficial effect on xylitol production. In spite of this, 34% of xylose was converted to xylitol in fed-batch fermentation by using non-growing cells, and a high 2.7 g $l^{-1}h^{-1}$ volumetric productivity of xylitol was achieved. This productivity level is not far from highest achieved in yeasts (4 - 5 g $l^{-1}h^{-1}$) (Nyyssölä et al. 2005).

4.4. Pyruvate

Pyruvate (pyruvic acid) is a three carbon α-ketocarboxylic acid that is used in the starting materials for the synthesis of amino acids such as L-tryptophan, L-tyrosine, L-phenylalanine and L-alanine (Causey et al. 2004, Li et al. 2001). Pyruvate is also used as a nutraceutical, a dietary supplement, and in the production of drugs and agrochemicals (Li et al. 2001, Li et al. 2002)

4.4.1. Production of pyruvate

Pyruvate is chemically synthesized from tartrate in an energy-intensive pyrolysis reaction (Zelić et al. 2004). Chemical synthesis additionally involves the use of toxic solvents (Causey et al. 2004). The biotechnical methods for the production of pyruvate involve purified enzymes, non-growing, immobilized or living cells (Li et al. 2001, Zelić et al. 2004). Fermentation is one of the most promising methods to produce pyruvate (Zelić et al. 2004).

Many microorganisms have been developed for the pyruvate fermentation, e.g. *Acinetobacter*, *Enterobacter*, *Enterococcus*, *Pseudomonas*, *Escherichia* and *Torulopsis* (Li et al. 2000). Currently, the yeast *Torulopsis glabrata* IFO 0005 (Miyta & Yonehara 1996) is used for the commercial pyruvate fermentation (Causey et al. 2004, Li et al. 2002). (Production of pyruvate in LAB is discussed in Study I).

5. AIMS OF THE STUDY

The aims of this study were to modify the metabolic pathways of lactic acid bacteria to produce industrially important compounds and characterize some essential and promising enzymes for metabolic engineering. The specific aims were:

1. To construct two types of inactivation mutants from *Lactobacillus fermentum* to produce mannitol and lactic acid or pyruvate. To obtain these mutants, deletions were targeted either at the *ldhD* gene or both the *ldhD* and *ldhL* genes.

2. To clone and express the mannitol dehydrogenase gene and to characterize its gene product from *Leuconostoc mesenteroides* ATCC 9135.

3. To construct and characterize a random mutant of *Leuconostoc pseudomesenteroides*, which is unable to grow on fructose and able to enhance mannitol production.

4. To clone, sequence and overexpress a xylitol-4-dehydrogenase gene from a gram-negative *Pantoea ananatis* ATCC43072 strain. The xylitol-4-dehydrogenase is a promising enzyme for the production of L-xylulose from xylitol.

6. MATERIALS AND METHODS

6.1. Bacterial strains, plasmids and growth conditions

All microbial strains, plasmids, growth medium and growth conditions used in this work are described in publications I-V.

6.2. Basic DNA techniques, transformation methods

Basic molecular biology techniques were used essentially as described by Sambrook and Russel (2001). Isolation of chromosomal DNA is described in Studies I-IV. DNA restriction and modification enzymes were used as recommended by the manufacturers (Promega, New England Biolabs Inc.). The polymerase chain reaction (PCR) was carried out using the reaction conditions recommended by the manufacturer (Finnzymes).

The Vectorette™ II system (Vectorette™ II: Sigma Genosys Ltd) (see chapter 6.3) was used to create genomic libraries of *L. fermentum* , *L. mesenteroides, L. pseudomesenteroides* and *P. ananatis* ATCC43072 and *P. ananatis* ATCC43073 according to the instructions of the manufacturer. Integration vectors for gene inactivations were constructed essentially as described before (Kylä-Nikkilä et al. 2000). Construction of *L. fermentum* Δ*ldhD* and Δ*ldhD*-Δ*ldhL* mutants is described in Study I and the cloning of *mdh*, *fruK* and *xdh* is described in Studies II – IV. Chemical mutagenesis of *L. pseudomesenteroides* ATCC12291 was performed as described in Study III. In subcloning, PCR fragments were cloned into the vector pGEM-T Easy (Promega) or pCR2.1-TOPO vector (Invitrogen).

Dot blot hybridisation was carried out essentially as described by Sambrook and Russel (2001). An *mdh* PCR probe was labelled with [α-^{33}P]dCTP using the High Prime labelling system (Roche). For detection and quantitation of hybridisation signals, the Molecular Imager System GS-525 and the Quantity One® program (Bio-Rad) were used. DNA sequencing was performed with the dideoxy chain termination method of Sanger et al. (1977) by using an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems) with the ABI PRISM® BigDye™ Terminators v2.0 or v3.1 Cycle Sequencing Kit (Applied Biosystems). DNA sequence analyses were performed using the Sequencer™ 3.0 program (Gene Codes Corporation). The transformation procedure for *L. fermentum* (Wei et al. 1995) was further optimized as described in Study (I). The transformation procedure for *L. lactis* and *E. coli* was performed essentially as described before by Holo and Nes (1989) and Sambrook and Russell (2001), respectively.

6.3. The Vectorette system

By using the Vectorette system it is possible to construct genomic libraries for isolating unknown gene(s). To isolate the target gene by genome walking, chromosomal DNA is cut with a chosen restriction enzyme (*Eco*RI, *Hin*dII, *Bam*HI, *Cla*I or some blunt restriction enzyme). These precut chromosomal DNA fragments are ligated to specific Vectorette units, which are partially double-stranded DNA structures having either

*Eco*RI, *Hin*dII, *Bam*HI, *Cla*I or blunt ends. The chromosomal DNA fragments and Vectorette units ligated together comprised the genomic Vectorette library, including the target gene(s), or fragments thereof.

An initiation PCR primer is designed from the known DNA sequence of target gene(s). Thus, by using the initiation PCR primer and a PCR primer specific for the Vectorette unit and the Vectorette library as a template, the unknown sequence between these primers is amplified by PCR. The amplification background is avoided because the complementary DNA strands of Vectorette units are partially mismatched. Therefore, the Vectorette PCR primer has no complementary strand to anneal in the first cycle of PCR. Only the initiation PCR primer will anneal to the strand and produce a complementary strand suitable for the Vectorette PCR primer. In the second cycle of PCR, both primers now have a template and PCR can continue normally. Furthermore, it is possible to sequence the obtained PCR product by using a Vectorette-specific sequencing primer, located further upstream in the Vectorette structure.

6.4. RNA isolation, Northern hybridization, primer extension and RT-PCR

For isolation of total RNA, *L. fermentum* and *L. pseudomesenteroides* cells were disrupted using glass beads in a mill homogenizer (Edmund Bühler) as described in Studies I and III, followed by RNA isolation with the RNeasy® Mini Kit (Qiagen). Total RNA from *L. mesenteroides* was isolated from the harvested protoplasts using the RNeasy® Mini Kit (QIAGEN). For isolation of total RNA, *P. ananatis* ATCC19321 and *P. ananatis* ATCC43072 cells were disrupted using glass beads in a FastPrep FP120 instrument (Thermo Savant) as described in Study IV and RNA was isolated with the RNeasy® Mini Kit (Qiagen) from the disrupted cells.

Northern hybridization was carried out as described in studies I-IV. The PCR probes for Northern analysis were labeled with [α-^{33}P] dATP or [α-^{33}P]dCTP using the Megaprime DNA Labeling System (Amersham Biosciences). The hybridisation signal was detected with the Molecular Imager System GS-525.

The 5´end determination of *mdh* and *fruK* transcripts was performed by using 5 pmol fluorescein-labelled specific oligonucleotides and an A.L.F. DNA Sequencer, as described earlier (Vesanto et al. 1995). For RT-PCR, RNA samples were treated with DNase I (Promega), purified with RNeasy columns (Qiagen) and reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the instructions of the manufacturers.

6.5. Protein and enzyme assays

Purification of xylitol dehydrogenase from *Pantoea ananatis* ATCC43072 is described in Study IV. Overexpression of mannitol dehydrogenase and xylitol-4-dehydrogenase in *E. coli* and their purification is described in Studies II and IV, respectively. The molecular mass of recombinant xylitol-4-dehydrogenase was estimated by analytical gel filtration using a HiLoad 26/60 Superdex 200 prep grade (Amersham Biosciences) column (2.6 by

32 cm) according to the instructions of the manufacturer. The protein concentration was determined with the Bio-Rad Protein Assay kit (Bio-Rad) according to the Bradford method (Bradford 1976). The N-terminus of the intact MDH and some of its tryptic peptides were sequenced using a gas-pulsed-liquid sequencer as previously described (Kalkkinen and Tilgmann 1988). The N-terminal amino acid sequence of the xylitol-4-dehydrogenase was determined at the Protein Chemistry Unit of the Biomedicum Center (Helsinki, Finland).

Mannitol dehydrogenase, lactate dehydrogenase, fructokinase and xylitol-4-dehydrogenase enzyme assays were performed as described in more detail in Studies I-IV. The D- and L-lactate concentrations were analyzed enzymatically using the D-Lactic acid/L-Lactic acid kit by Boehringer Mannheim (catalogue no. 111 2821).

6.6. Bioreactor cultivations and analysis

Bioreactor cultivations are described in Studies I, III and IV. Concentrations of organic acids, sugars, ethanol, and mannitol were determined by high-performance liquid chromatography (HPLC). Purification of xylulose for polarimetric analysis is described in Study IV.

7. RESULTS AND DISCUSSION

7.1. Cloning and sequencing of the *ldhD* and *ldhL* gene regions from *Lactobacillus fermentum* NRRL-B-1932 (Study I)

A heterofermentative *Lactobacillus fermentum* NRRL-B-1932 strain can convert fructose almost quantitatively to mannitol when glucose is used as a co-substrate. In addition to mannitol, it produces a mixture of D- and L-lactate, acetate and some ethanol. Commercial use of this acidic mixture is difficult, since separation of the acids is not a cost effective process. In this study, the problem has been approached by aiming to develop a two-product concept. With deletion of either or both of the D- and L-lactate dehydrogenase genes (*ldhD* and *ldhL*) from a mannitol producing *L. fermentum* strain the aim has been to create an organism that produces mannitol and either pure L-lactate or pyruvate in a single process.

The *L. fermentum* D- and L-lactate dehydrogenase genes (*ldhD* and *ldhL*) were isolated by PCR amplification using degenerated oligonucleotides designed according to the conserved DNA regions of the *ldh* gene sequences from different LAB (Figure 7). A 0.7-kb PCR-fragment was generated from both the *ldhD* and *ldhL* genes. Their sequence was found to share a high similarity with the *ldhD* and *ldhL* genes of other bacteria. For inactivation constructs, the upstream regions of the *ldh* genes were isolated using the Vectorette system followed by sequencing of these 1-kb regions found for *ldhD* and *ldhL*.

7.1.1. Inactivation of the *L. fermentum ldhD* gene

The inactivation of *ldhD* was carried out with the integration vector pKTH5096 in such a way that a 0.4-kb fragment from the promoter and the 5´-region of *ldhD* was deleted during the gene replacement. After the second homologous recombination, one clone designated *L. fermentum* GRL1030 showed a D(-)-lactic acid negative phenotype. The correct size of the *ldhD* deletion and absence of the integration vector was confirmed by PCR. Inactivation of the *ldhD* gene in *L. fermentum* GRL1030 was also verified as the absence of the *ldhD* transcripts by Northern blotting.

7.1.2. Co-production of D-mannitol and L-lactate by the *L. fermentum* Δ*ldhD* strain GRL 1030

In bioreactor cultivations, the single *ldhD* gene mutant GRL1030 produced mannitol and L-lactic acid, as expected. Mannitol and lactic acid yields and productivities were practically unaffected by the deletion of the *ldhD* gene. The primary fluxes of the mutant did not differ significantly from the respective fluxes of the parent strain. No novel end products were detected. The mutant cells, however, grew and consumed fructose slightly slower than the parent cells. Surprisingly, the L-lactate dehydrogenase activity of the mutant cells was reduced to about 1/3 of that of the parent strain.

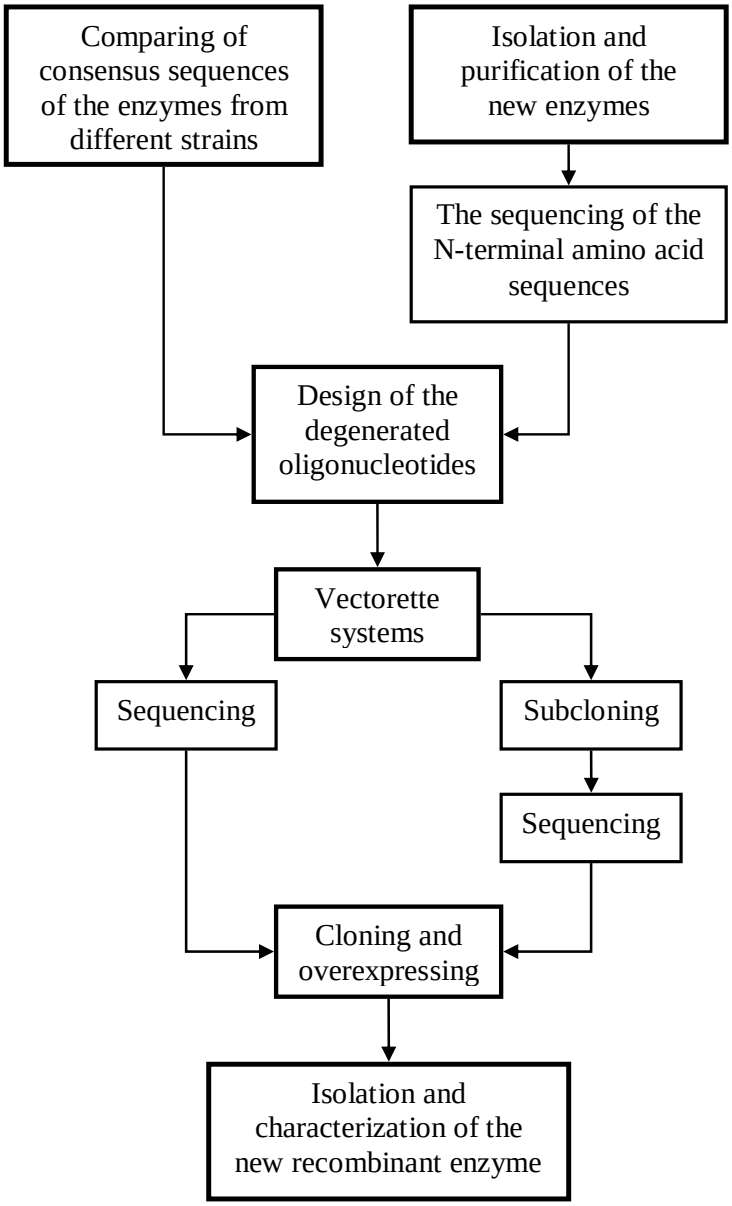

Figure 7. Schematic representation of the isolation and cloning of a new enzyme.

The mutation also resulted in small changes in the NADH oxidation patterns. While slightly less mannitol was produced per unit fructose consumed by the mutant, a small increase in ethanol production was observed. Moreover, the yield of ATP per unit fructose consumed was lower in the D-lactate dehydrogenase negative mutant than in the parent strain (86.7 mmol/mol compared to 96.3 mmol/mol). This corresponded well with the lowered growth rate and final biomass of the mutant.

The use of metabolic engineering for the production of pure L-lactic acid has previously been studied in homofermentative LAB. For instance, using *Lactobacillus helveticus*, Bhowmik and Steele (1994) and Kylä-Nikkilä et al. (2000) constructed D-lactate dehydrogenase negative mutants, which produced only pure L-lactate. The metabolism of these mutants was otherwise unchanged. Lapierre et al. (1999) inactivated the D-lactate dehydrogenase gene in *Lactobacillus johnsonii*, which resulted in the production of pure L-lactate, but now some pyruvate was lost to other end products (e.g. diacetyl and acetoin). In these *L. johnsonii* mutant strains, a decrease in the remaining L-lactate dehydrogenase activity was also detected.

7.1.3. Inactivation of the *ldhL* gene of *L. fermentum* Δ*ldhD* strain GRL1030

To construct a Δ*ldhD*-Δ*ldhL* double mutant for *L. fermentum*, the *ldhL* inactivation vector pKTH5097 was transferred to the D-lactate dehydrogenase negative mutant strain GRL1030. The gene replacement resulted in a 0.4-kb deletion at the promoter and 5´-region of *ldhL*. After the second homologous recombination an L (+)-lactic acid negative phenotype was found and designated *L. fermentum* GRL1032. PCR analysis of GRL1032 DNA with *ldhL* and *ldhD* specific oligonucleotides further confirmed that the size of both deletions was correct, and no PCR amplification products were detected either from the Δ*ldhL* and Δ*ldhD* deleted regions or the integration vector. Inactivation of the *ldhL* and *ldhD* genes in *L. fermentum* GRL1032 was also verified as the absence of the *ldhL* and *ldhD* transcripts by Northern blotting.

7.1.4. Co-production of D-mannitol and pyruvate by the *L. fermentum* Δ*ldhD*-Δ*ldhL* mutant strain GRL1032

The hypothesis was that a GRL1032 Δ*ldhD*-Δ*ldhL* double mutant would excrete pyruvate to the growth medium, thus giving rise to a novel microbial two-product process (D-mannitol and pyruvate). To maximize the amount of ionized pyruvate in the medium and to prevent the free backflow of nonionized acid, the pyruvate fermentation was performed at pH 7.0.

The double mutant produced mannitol and pyruvate as expected. In addition to pyruvate, the double mutant produced 2,3-butanediol. More surprisingly, some lactic acid was still produced.

The sugar consumption patterns of the double mutant deviated clearly from the respective patterns of the *L. fermentum* parent strain. The glucose consumption of the double-mutant GRL1032 was severely depressed compared to the fructose consumption. The lack of lactate dehydrogenases drastically affected the glucose catabolism and the redox balance of the cells, resulting in reduced cellular growth. When the initial fructose (20 g/L) was consumed by the mutant cells ($t \approx 7$ h), only about 55% of the initial glucose (10 g/L) was consumed. After fructose was depleted in the mutant cell cultivations, the cells consumed glucose very slowly.

Importantly, similar mannitol production levels were obtained with both strains. However, although the yield of mannitol from fructose remained high, its volumetric productivity was reduced. Due to the lack of the lactate dehydrogenases, pyruvate accumulated into the growth medium (2.0 g/L). 2,3-Butanediol was also produced from pyruvate (0.4 g/L). To fulfill the remaining redox imbalance, a small amount of ethanol was produced, whereas acetyl-P was mainly de-phosphorylated into acetate. In this work, it has been assumed that NAD^+, instead of $NADP^+$, is the cofactor of the key enzymes.

In contrast to expectations, the D/L-lactate dehydrogenase negative mutant also produced some D-lactate (0.5 g/L compared to 6.4 g/L of total lactate produced by the parent strain). The formation of lactate in the mutant cultivations was puzzling. The inactivation of the lactate dehydrogenase genes had been checked thoroughly: First, no lactate dehydrogenase activities were detected in cell lysates from mutant cell cultures. Second, using PCR and several different sets of primers, it was ensured that correct inactivation had occurred on the DNA level. Third, applying Northern blotting, the mRNAs encoding for both lactate dehydrogenases clearly detected in the parent cells were not detected in the mutant cells. Although the lactic acid could be produced from media components, e.g. from citrate by malolactic fermentation or from leucine, the accuracy of the carbon- and redox balances supported the fact that the lactate formed was actually produced from pyruvate.

In *Lactobacillus plantarum*, an *ldh*-negative mutant was also found to produce small amounts of lactic acid, which was suggested to be due to a D-hydroxyisocaproate dehydrogenase activity (Ferain et al. 1996). This enzyme, related to D-lactate dehydrogenases, has been detected in several lactic acid bacteria. However, we could not detect this activity from the double *ldh*-mutant strain under the assay conditions used. Thus, the formation of D-lactate could not be explained, but it was speculated that activation of another enzyme, with D-lactate dehydrogenase side-activity, was responsible for reducing a fraction of pyruvate to lactate in these cells. Recently, some alternative sources for Ldh-activities have been found in LAB. An *L. lactis* strain whose *ldhL* was disrupted accumulated lactate by activating a silent *ldhB* gene coding for an Ldh protein (LdhB) (Bongers et al. 2003). Also, inactivation of the genes encoding the lactate dehydrogenase activities in *Lactobacillus casei* did not abolish the production of L-lactate, suggesting the presence of at least a second L-Ldh (Viana et al. 2005). However, these observations do not explain the lactic acid found in our experiment, since we could not find any Ldh activity from the mutant strain or any sequence homology in Northern blot and PCR experiments.

In earlier studies with homofermentative/facultative heterofermentative LAB, the inactivation of both lactate dehydrogenases in *Lactobacillus plantarum* resulted in major rerouting of glucose catabolism, which led to the production of novel end products such as acetoin, ethanol, acetate, mannitol and succinate (Ferain et al. 1998). Moreover, Neves et al. (2000) studied the disruption of the lactate dehydrogenase gene in *L. lactis*. In a resting state, the mutant cells produced the same novel end products as above. Mannitol was transiently produced and metabolized once glucose was depleted. In these cells, mannitol was formed from fructose-6-P via mannitol-1-P.

As described earlier, in normal conditions *L. lactis* do not produce mannitol. However, Gaspar et al. (2004) and Wisselink et al. (2004, 2005) have succeeded in constructing some *L. lactis* strains that produce mannitol from glucose. Wisselink et al. (2005) achieved the highest conversion yield from glucose to mannitol (50%) with an *L. lactis* LDH deficient strain that overexpressed the mannitol 1-phosphate dehydrogenase gene (*mtlD*) of *L. plantarum* together with a mannitol 1-phosphate phosphatase of *Eimeria tenella* (a protozoan parasite). This 50% yield was obtained by using growing cells and it is close to the theoretical mannitol yield of 67% in *L.lactis* (Wisselink et al. 2005). The advantage of *L. lactis* in mannitol production compared to heterofermentative LAB is its capability to use several sugar substrates to synthezise mannitol (Wisselink et al. 2005). However, the volumetric mannitol productivity of these *L. lactis* strains appears to be much lower than in heterofermentative LAB.

In conclusion, in the *L. fermentum* Δ*ldhD*-Δ*ldhL* mutant strain at pH 7.0, 38.5 g fructose (213.7 mmol) and 11 g glucose (61.9 mmol) were metabolized to 34 g D-mannitol (188.2 mmol) and 4 g pyruvic acid (44.3 mmol) in 7 hours. Hence, in this process about 76.3% (C-mol/C-mol sugar) of the total carbon present in sugars was recovered in valuable end products (D-mannitol and pyruvic acid). Similar levels were earlier obtained with the mannitol and L-lactic acid two-product process. However, the concentration of pyruvate obtained was not as high as expected, which complicates the purification steps. The low level of pyruvate formed was primarily due to the severe slow-down of glucose catabolism or transport and the formation of "waste products" such as 2,3-butanediol and lactate.

7.2. Isolation of the *Leuconostoc mesenteroides* ATCC9135 mannitol dehydrogenase and cloning its gene (Study II)

7.2.1. Isolation of the mannitol dehydrogenase

The mannitol dehydrogenase (MDH) enzyme catalyzes the NAD(P)H-dependent reduction of D-fructose to D-mannitol. It is a promising enzyme for overexpressing in the various LAB for enhanced production of mannitol. In this study, the mannitol dehydrogenase gene (*mdh*) has been isolated from *Leuconostoc mesenteroides* ATCC9135 and the enzymatic properties of its gene product, MDH, has been characterized. MDH enzymes have previously been purified from *Lactobacillus brevis* and *L. mesenteroides* (Martinez et al. 1963, Sakai & Yamanaka 1968a, b). After this work was completed, cloning or/and characterization of MDH enzymes from *Lactobacillus sanfranciscensis* (Korakli & Vogel 2003), *Leuconostoc pseudomesenteroides* (Hahn et al. 2003), *Lactobacillus intermedius* (Saha 2004), *L. brevis* (Liu et al. 2005) and *Lactobacillus reuteri* (Sasaki et al. 2005) have been reported.

The N-terminal amino acid sequences of the intact protein and three tryptic peptides were determined from a 41 kDa protein purified from a commercial MDH enzyme preparation of *Leuconostoc mesenteroides* ATCC-9135. Using degenerated oligonucleotides deduced from these peptide sequences, a 700-bp PCR product from the putative mannitol

dehydrogenase (*mdh*) gene was obtained. The Vectorette system was used to isolate the missing part of the gene region encoding the whole 41 kDa protein (Figure 7).

7.2.2. Sequence analysis of the *L. mesenteroides mdh* gene

The sequence analysis of the 2.5-kb gene region isolated revealed the putative *L. mesenteroides mdh* gene as an open reading frame (ORF1) of 1,014 bp with an encoding capacity for a protein of 338 amino acids. The amino acid sequences determined for the intact N-terminus and tryptic peptides of the 41 kDa protein were found from the predicted amino acid sequence of ORF1. A putative promoter with the -35 and -10 regions could be identified 41 bp upstream of the ATG start codon of *mdh*. A conserved region for a putative ribosome binding site (Shine & Dalgarno 1974) located 8 bp upstream of the start codon of *mdh*. No transcription terminator sequence could be recognised within 1,180 nucleotides downstream of the stop codon of the *mdh* gene.

7.2.2.1. Homology analysis of the MDH

Homology searches revealed that the predicted amino acid sequence of the *L. mesenteroides* MDH shared 93% and 73% overall identity with MDHs from *L. pseudomesenteroides* and *L. reuteri*, respectively. However, homology with that of other MDHs from various micro-organisms was negligible (overall identity below 17%). These other microbial mannitol dehydrogenase genes (*mdh*) characterised from *Rhodobacter sphaeroides, Pseudomonas fluorescens* and *Saccharomyces cerevisiae,* or identified from gene libraries, belong to a heterologous family of long-chain dehydrogenases and show a sequence identity of 30-40% with each other (Brünker et al. 1997, Kulbe et al. 1987, Schneider & Giffhorn 1989, Schneider et al. 1993).

However, significant identity (30-33%) was also found between the *L. mesenteroides* MDH and other dehydrogenases, such as a putative zinc-containing alcohol dehydrogenase protein from *Sinorhizobium meliloti*, a putative zinc-binding dehydrogenase of *Streptomyces coelicolor*, a putative alcohol dehydrogenase from *P. aeruginosa* and a sorbitol dehydrogenase of *Staphylococcus aureus*. These enzymes are members of the dehydrogenase subgroup of the medium-chain dehydrogenase/reductase superfamily (MDR). The putative NAD^+ binding site (Wierenga et al. 1985) was found in the amino acid sequence of the *L. mesenteroides* MDH. The sequence motif and the location and spacing of coenzyme binding residues common to all medium-chain dehydrogenases were also found in *L. mesenteroides* MDH (Martínez et al. 1996, Schneider et al. 1993). Furthermore, sequence alignment also revealed a number of conserved residues, many of them glycine residues, in addition to those involved in co-factor binding, suggesting a conserved three dimensional structure (Luque et al. 1998).

In addition, hybridisation testing of the *L. mesenteroides mdh* gene probe with *L. brevis, L. plantarum, L. helveticus, L. lactis, L. mesenteroides, L. pseudomesenteroides, L. fermentum, P. fluorescens* and *E. coli* was performed. Quantitation of the hybridisation signals showed that the *L. mesenteroides mdh* probe hybridised strongly with *L. pseudomesenteroides* chromosomal DNA and weakly with *L. brevis* and *L. fermentum*

DNA, whereas with the rest of the heterologous DNAs tested the hybridisation result was negative. These hybridisation data suggest that obligate heterofermentative LAB may share a similar *mdh* structure. The *L. mesenteroides mdh* gene was the first *mdh* sequence isolated from heterofermentative LAB and the sequence and hybridization data suggested that MDH of obligate heterofermentative LAB form a new group of MDH enzymes distinct from other bacterial MDHs. This was later confirmed when new *mdh* genes were isolated from other heterofermentative LAB. In other LAB, such as homo-/facultative heterofermentative *L. lactis* and *L. plantarum*, mannitol phosphate dehydrogenase (MPDH) is used for mannitol synthesis instead of MDH (Chakravorty 1964, Gaspar et al. 2004).

7.2.2.2. Sequence analysis of the ORF2

Two nucleotides downstream of the putative *mdh* gene (ORF1) another open reading frame (ORF2) of 735 bp with a coding capacity for an unknown protein of 245 amino acids was found. Sequence analysis of ORF2 suggested that it is an integral membrane protein, with five predicted transmembrane segments. The second gene (ORF2) of the operon did share 40% identity to a putative sugar-specific permease of *L. plantarum*. The predicted amino acid composition of the ORF2 encoded gene product also suggests a transmembrane location for this protein. Whether the function of ORF2 encoded protein is associated with that of MDH cannot be deduced from the available data.

7.2.2.3 Analyses of *L. mesenteroides mdh* transcripts

Northern blot analysis of total RNA isolated from *L. mesenteroides* revealed an approximately 2.2-kb transcript with an *mdh* specific probe, indicating that *mdh* is transcribed as a polycistronic mRNA. Determination of the 5´ end of the 2.2-kb transcript located the transcription start site (T) 35 nucleotides upstream of the initiation codon, which is in agreement with the suggested putative promoter sequence.

7.2.3. Overexpression of *mdh*

For the production and purification of MDH, the *L. mesenteroides mdh* gene was tagged with six histidine codons in the overexpression vector pKK223-3, resulting in plasmid pKTH5123, which was expressed in *E. coli* M15. The active mannitol dehydrogenase was isolated using HisTrap purification. The purified MDH fraction appeared in SDS-PAGE as single band of 41 kDa. Purified recombinant MDH gave a specific activity of 70 U MDH/mg of protein with a high purification factor of 100 and a yield of 37%. As compared to the high expression level after IPTG induction, it is likely that part of MDH in *E. coli* was either inactive or occurred as aggregates. Thus, further control of the induction level and conditions are likely to increase the yield of purified active MDH. During the isolation and purification process a high dependence on the presence of reducing agent was also observed, suggesting a central role of the cysteine residues in the structural stability of MDH. Whether overexpression of the *L. mesenteroides mdh* gene in *L. mesenteroides* or some other heterofermentative LAB will increase the mannitol production awaits testing.

7.2.4. Characterisation of the enzymatic properties of MDH

Assays with the purified MDH activity at different mannitol and fructose concentrations showed a K_m of 32 mM and 71 mM, respectively. In substrate specificity tests with a variety of sugar alcohols, ketohexoses and ketopentoses, only D-fructose 1-phosphate showed activity (11% relative activity) in addition to mannitol and fructose.

The kinetic properties and substrate specificity obtained in the present study with the recombinant MDH enzyme were in good agreement with the published data. Both data also confirmed the high specificity of the *L. mesenteroides* MDH enzyme for fructose and mannitol. Mannitol dehydrogenase enzymes (MDH) characterized from *P. fluorescens* and *R. sphaeroides*, which belong to a heterologous family of long-chain dehydrogenases, are instead polyol dehydrogenases with broad substrate specificity (Brünker et al. 1997, Schneider et al. 1993, Slatner et al. 1999).

MDHs purified from heterofermentative lactic acid bacteria, *L. brevis* (Martinez et al. 1963), *L. pseudomesenteroides* (Hahn et al. 2003), *L. sanfranciscensis* (Korakli & Vogel 2003), *Lactobacillus intermedius* (Saha 2004) and *L. reuteri* (Sasaki et al. 2005) are also highly specific for fructose and mannitol. However, the structure and cofactor specificity differ between these heterofermentative LAB MDHs (Table 1). *L. mesenteroides* (Yamanaka et al. 1977) and *L. pseudomesenteroides* (Hahn et al. 2003) MDHs have been found to consist of four presumably identical subunits of about 38 kDa and 43 kDa, respectively, and have NADH as a cofactor. However, *L. reuteri* MDH, which has 76–77% amino acid sequence identity with these *Leuconostoc* MDHs, was found to be a dimer with subunits of 40 kDa in size (Sasaki et al. 2005). *L. brevis* MDH also has about 75% amino acid sequence identity with *Leuconostoc* MDHs but is able to use both NADH and NADPH as the cofactor (Liu et al. 2005, Martinez et al. 1963). The MDH of *L. sanfranciscensis* showed an apparent molecular mass of 44 kDa with a monomer structure (Korakli & Vogel 2003). *L. intermedius* (Saha 2004) MDH was heterotetrameric and consisted of two subunits of 43 kDa and 34.5 kDa in size. The two last *Lactobacillus* MDHs preferred NADPH as the cofactor (Korakli & Vogel 2003, Saha 2004).

It is feasible that in heterofermentative LAB the energetic advantage of using fructose as an alternative electron acceptor, resulting in mannitol production, has led to the evolution of a specific *mdh* gene in these bacteria.

Table 1. Properties of some bacterial mannitol dehydrogenases

	Molecular structure	Molecular mass kDa		Cofactor	K_m for fructose (mM)	Identity to *L. mesenteroides* (%)	Reference
		Subunit	Native[(1)]				
L. mesenteroides	tetramer	36[(4)]	137	NADH	35	100	Yamanaka et al. 1977, this study
L. pseudomesenteroides	tetramer	36[(4)]	155	NADH	44	93	Hahn et al. 2003
L. reuteri	dimer	36[(4)]	75	NADH	34	73	Sasaki et al. 2005
L. brevis	No data	36[(4)]	No data	NADH/ NADPH	70	75.4	Liu et al. 2005, Martinez et al. 1963
L. sanfranciscensis	monomer	44[(2)]	53	NADPH	24	No data	Korakli & Vogel 2003
L. intermedius	heterotetramer	43, 34.5[(2)]	171	NADPH	25	No data	Saha 2004
P. fluorescens	monomer	54.5[(4)]	45 ± 5[(3)]	NADH	25	16	Brünker et al. 1997, Schneider et al. 1993

[(1)]Determined by using gel chromatography, [(2)] by SDS-PAGE. [(3)] Native molecular mass determined from recombinant *E. coli* strain. [(4)]Deduced from DNA sequence data.

7.3. Isolation of *Leuconostoc pseudomesenteroides* mutants unable to grow on fructose (Study III)

7.3.1. Inactivation of fructokinase activity by random mutagenesis

Fructokinase (FK) phosphorylates intracellular fructose into fructose-6-phosphate. The fructose-6-phosphate is further isomerized to glucose-6-phosphate by phosphoglucose isomerase (PGI) and channeled into the PK pathway. According to the hypothesis of this study, the inactivation of a gene encoding one of these enzymes would prevent the leakage of fructose into the PK pathway and give an improved yield of mannitol from fructose. Therefore, we aimed at improving the mannitol production of *L. pseudomesenteroides* by constructing mutants lacking fructokinase activity. Unfortunately, the single crossover integration with pG^+host4 did not succeed and thus did not allow the use of directed inactivation techniques on *L. pseudomesenteroides*. Therefore, inactivation of the fructokinase activity from *L. pseudomesenteroides* was performed with chemical mutagenesis followed by screening of mutants unable to grow on fructose.

Mutants of *L. pseudomesenteroides* ATCC12291 that were unable to grow on fructose were induced by chemical mutagenesis (MNNG). The mutant with the lowest fructokinase activity (10% of that of the parent strain), named BPT143, was selected for further studies. The fructose uptake and mannitol dehydrogenase activity of this mutant were unaltered and still allowed fructose to be converted into mannitol when glucose was present in the growth medium.

7.3.2. Sequence analysis of the *L. pseudomesenteroides* fructokinase gene (*fruK*)

7.3.2.1. Isolation of the *fruK* gene

To characterize the fructokinase gene (*fruK*) from the wild type *L. pseudomesenteroides* ATCC12291, the gene was isolated by PCR amplification using two degenerated oligonucleotides. These degenerated oligonucleotides were designed according to the conserved regions of fructokinase genes from various LAB and *B. subtilis* (Figure 7). Sequence analysis of the amplified 0.5 kb PCR fragment revealed high similarity with other bacterial fructokinases. The unknown upstream (0.5 kb) and downstream (0.8 kb) regions of the *fruK* gene were isolated using the Vectorette II system and specific inverse primers, followed by sequencing of the obtained *fruK* fragments.

The coding sequence of the *L. pseudomesenteroides fruK* gene was found to be 862 bp in size and preceded by a putative -35 and -10 promoter region starting 94 base pairs upstream of the translation initiation site. A *cre*-like consensus sequence (Inacio et al. 2003) was found in the promoter region. A putative ribosome binding site was located six base pairs upstream of the translation initiation codon. A putative stem-loop transcription terminator with ΔG = -84 kJ was located 290-325 bp downstream of the translation stop codon. In addition, an upstream stem-loop terminator with ΔG = -84 kJ was found 490 - 453 bp upstream of the translation initiation codon of *fruK*. No additional open reading frames were found 450 bp upstream or 460 bp downstream of the *fruK* coding sequence by the sequence analyses performed. Homology searches revealed that the predicted amino acid sequence of the *L. pseudomesenteroides* fructokinase shared 51-56% identity with fructokinases from *Clostridium acetobutylicum*, *Clostridium beijerincki*, *Enterococcus faecalis* and *Listeria monocytogenes*.

7.3.2.2. Analyses of *fruK* transcripts

Expression data from BPT143, revealing an absence of *fruK* transcripts, was in accordance with the reduced fructokinase activity of the mutant. The size of the hybridized *fruK* transcript of wild-type *L. pseudomesenteroides* was determined to be approximately 1.3 kb. Determination of the 5´-end of the 1.3 kb transcript, located at the transcription start site seven nucleotides downstream of the -10 region of the *fruK* promoter and 59 nucleotides upstream of the initiation codon. According to these results and the transcription terminator analysis, the actual size of the *fruK* transcript was verified to be 1257 bp. Our results with the *L. pseudomesenteroides fruK* gene showing that *fruK* is monocistronic and flanked by approximately 350 bp long inverted repeats (IR) (Figure 8), suggests that the ability of *L. pseudomesenteroides* to use unphosporylated intracellular fructose has evolved via lateral Tn-transposition during evolution.

Figure 8. Schematic representation of the *L. pseudomesenteroides* ATCC12291 *fruK* gene and its flanking areas. The coding sequence is marked with *fruK*, the promoter area with P and inverted repeat sequences with IR. Transcription terminators are marked with hairpin loops.

7.3.2.3. Sequence analysis of mutation

The possible mutations in the *fruK* gene sequence of fructose-negative strain BPT143 were analyzed by sequencing the *fruK* gene. A silent point mutation in Thr104 was identified in the *fruK* sequence of the mutant. This was not, however, likely to be the reason for the reduced fructokinase activity. No mutations could be found in the 450 bp upstream region of *fruK*, suggesting that the putative *fruK* promoter sequence was not directly affected by the mutagen. It would therefore seem likely that some more distant sequences, involved in the regulation of *fruK* transcription, were mutated. For example, a regulatory protein may have been affected by the mutagen. In an activator-based system, inactivation of this protein or its synthesis would have directly resulted in the phenotype observed. On the other hand, if the *fruK* gene is controlled by a repressor, e.g. either a constitutive repressor mutation or a non-inducible repressor mutation, this could then result in the phenotype found. It is not possible to completely rule out DNA conformation rearrangement effects as the cause of the change in the BPT143 phenotype

7.3.3. Bioreactor cultivations

The effect of the random mutation on mannitol production was also studied in bioreactors under process conditions. Comparison of some key properties of wild type *L. pseudomesenteroides* ATCC12291 and its fructokinase mutant BPT143 revealed that the mutant grew faster than the parent strain. More importantly, the specific fructokinase activity was significantly reduced in the mutant, which decreased channeling of fructose into the PK pathway, resulting in an improved mannitol yield (from 74 to 86 mol%). A faster fructose consumption rate and the improved mannitol yield subsequently resulted in a slightly better volumetric productivity of mannitol by the mutant (from 2.1 to 2.8 g l^{-1} h^{-1}).

Both the parent strain and the mutant consumed approximately the same amount of glucose in relation to fructose. Due to a greater leakage of fructose into the PK pathway, the parent cells produced clearly more carbon dioxide, lactic acid and ethanol than the mutant cells did. To balance the increased NADH oxidation due to increased mannitol production, the mutant cells produced less ethanol and more acetic acid.

It has been reported for some native LAB strains that only negligible amounts of fructose leak into the PK pathway when grown in a 2:1 mixture of fructose and glucose (e.g. Korakli

et al. 2000, Saha & Nakamura 2003). This is usually achieved at a low pH. However, acidic process conditions result in decreased cell metabolism, which subsequently cause decreased volumetric mannitol productivity. A fructokinase-negative mutant could enable a higher pH to be used in the production process without lowering the yield. With such a mutant both yield and productivity could be maximized. Despite the 90% decrease in fructokinase activity a 100% yield of mannitol from fructose was not achieved. A possible explanation for this is that the fructose phoshorylation pathway is simply more efficient than the fructose reduction reaction, which makes it difficult to completely direct the flux to mannitol. Nevertheless, our results indicate that the yield can be improved by modifying the fructokinase. It is possible that a quantitative yield would be achieved if the fructokinase activity was completely disrupted and the mutant strain did not have a PTS transporter for fructose.

7.4. Isolation of xylitol-4-dehydrogenase from *Pantoea ananatis* ATCC43072 and cloning of the corresponding gene (Study IV)

Xylitol-4-dehydrogenase (XDH) catalyzes the oxidation of xylitol to L-xylulose. This enzyme has the potential to produce L-xylulose in recombinant LAB. In this study, a novel xylitol-4-dehydrogenase gene has been cloned and characterized from gram-negative *Pantoea ananatis* ATCC43072, a mutant strain capable of growing with xylitol as the sole carbon source. The *xdh* gene was further overexpressed in *E. coli* and the XDH enzyme was purified to homogeneity and its enzymatic properties were characterized.

7.4.1. Isolation of xylitol-4-dehydrogenase from *Pantoea ananatis*

Xylitol-4-dehydrogenase was isolated from *P. ananatis* ATCC43072 cell extract. The N-terminal amino acid sequence of the purified 30 kDa protein was determined and a degenerated oligonucleotide deduced from this peptide sequence was used to isolate the xylitol-4-dehydrogenase gene (*xdh*) from *P. ananatis* gene Vectorette libraries (Figure 7). Using *P. ananatis* ATCC43073 *Hin*dIII Vectorette amplicons as a template, three PCR products of different sizes (0.6, 1.0 and 1.8 kb) were obtained. Two (0.6 and 1.0 kb) of these PCR products were cloned to the pCR2.1-TOPO vector using *E. coli* TOP10 as the host and sequenced with vector specific primers. The 1.0-kb fragment appeared to contain part of the putative xylitol dehydrogenase gene (*xdh*). To isolate and sequence the rest of the *xdh* gene, *Eco*RI and *Hin*dIII digested *P. ananatis* ATCC43072 Vectorette amplicons were used.

7.4.2. Sequence analysis of the *P. ananatis* ATCC43072 *xdh* gene

Sequence analysis of the 2.1-kb gene region isolated revealed the putative *P. ananatis xdh* gene as an open reading frame (ORF1) of 795 bp with an encoding capacity for a protein of 264 amino acids. The amino acid sequence determined from the N-terminus of the intact 30 kDa protein was found from the predicted amino acid sequence of ORF1. In addition, three other sequences derived from ESI-MS/MS analysis of XDH fragments corresponded to the amino acid sequence deduced from ORF1.

The 5´-end region of another open reading frame (ORF2) encoding an unknown protein was found 54 nucleotides downstream of the putative *xdh* gene (ORF1). Two putative promoters with the -35 and -10 regions could be identified 18- and 302- bp upstream of the ATG start codon of *xdh*. A conserved region for a putative ribosome binding site (Shine & Dalgarno 1974) was localized 6 bp upstream of the start codon of *xdh*. No transcription terminator sequence could be recognized within the available sequence downstream of the translation stop codon of the *xdh* gene.

7.4.2.1. Homology analysis of the XDH

Homology searches revealed that the predicted amino acid sequence of the *P. ananatis* XDH shared significant identity (38-51%) with members of the classical short-chain dehydrogenase/reductase family (SDR), e.g. with an oxidoreductase from *Brucella abortus*, a 2-deoxy-D-gluconate 3-dehydrogenase from *Burkholderia mallei,* and a dicarbonyl/L-xylulose reductase from *Cavia porcellus* (Guinea pig). The SDR superfamily forms a large and functionally heterogeneous protein family, in which 3D structures display highly similar α/β folding patterns (Kallberg et al. 2002, Oppermann et al. 2003). SDR has five families from which the classical and extended families are the largest ones. The classical family has been further divided into seven subfamilies (Kallberg et al. 2002, Persson et al. 2003).

Similar secondary structure elements common to all classical short-chain dehydrogenases were found in *P. ananatis* XDH. Furthermore, the putative coenzyme binding site and amino acid residues located in the active sites of these enzymes were found from the same secondary structure elements (Kallberg et al. 2002). However, the conserved amino acid residue (D/E) at the end of second β strand at the beginning of the Rossmann fold, which determines different coenzyme-binding subfamilies (Kallberg et al. 2002, Persson et al. 2003), could not be found in XDH, thus preventing the further determination of the subfamily of *P. ananatis* XDH. Surprisingly, in spite of the conserved overall structure, the cysteine residues, which have strong impact on the XDH activity, show no conservation regarding the primary sequence or the secondary structures. The available amino acid sequence of the truncated ORF2 shared significant identity (40-47%) with the ribose/sugar-binding protein of ABC transporters from *Rhizobium loti*, *Yersinia pestis, Brucella abortus* and *E. coli*.

7.4.2.2. Analysis of *P. ananatis xdh* transcripts

Analysis of *xdh* derived transcripts was hampered by the high instability of these mRNAs. Thus, only degradation products could be detected. Northern blot analysis of xylitol-positive mutant *P. ananatis* ATCC43072 revealed repeatedly in independent experiments the same cluster of the transcripts of approximately 1, 2.2 and 3-kb in size when using an *xdh*-specific probe, suggesting that *xdh* is transcribed as a part of polycistronic mRNA. These transcripts were found only when *P. ananatis* ATCC43072 was grown with xylitol as the sole carbon source. If the cells were grown either with glucose or a mixture of glucose and xylitol as the carbon sources, no *xdh*-specific

transcripts were obtained. Northern blot analysis of the wild type strain *P. ananatis* ATCC19321 grown on glucose revealed no *xdh* transcripts. A previous report has indicated that *xdh* is constitutively expressed in the ATCC43072 mutant strain (Doten & Mortlock 1985a). This is in contrast to our results, which show that *xdh* transcription is strictly repressed in the presence of glucose regardless of the presence of xylitol in the growth medium. No *cre*-like consensus sequence (Inacio et al. 2003) was, however, found within the promoter region sequenced. It remains to be tested whether carbon sources other than glucose could allow transcription of the *xdh* operon without the presence of xylitol.

To further analyze the location of *xdh* in an operon structure, the *xdh* transcript of *P. ananatis* ATCC43072 was subjected to RT-PCR. Using different primer pairs and reverse transcribed cDNA as the template, the RT-PCR analysis revealed that *xdh* and the truncated ORF2 most likely belong to the same operon, which is also in accordance with the DNA sequence data and Northern analyses. RT-PCR also suggested that *xdh* is the first gene of this operon. As indicated in the results, the gene product of ORF2 has significant similarity to the ribose/sugar-binding protein of ABC transporters. If the 3.0-kb *xdh*-ORF2 transcripts indeed represent the entire operon, the size of this mRNA excludes the possibility of encoding other putative components of the expected ABC transporter. Whether the ORF2-encoded protein and XDH are functionally related remains to be established.

7.4.2.3. Analysis of *P. ananatis xdh* mutations

To identify the putative mutations that had converted the wild type, XDH-negative phenotype of *P. ananatis* ATCC19321 to the XDH positive phenotype of *P. ananatis* ATCC43072, reported previously (Doten & Mortlock 1985b), the *xdh* gene and its upstream region were analyzed by PCR and DNA sequencing from both strains. Sequencing of the PCR amplification product covering the putative promoter region and 450 bp downstream from the initiation codon of *xdh* revealed no differences between the wild type strain and the mutant. Furthermore, no structural differences between the strains could be found downstream of *xdh* by PCR analysis. Instead, a putative mutation site could be localized by PCR analysis upstream of the 350 bp region as a lack of PCR products with the wild type DNA when the upstream specific mutant strain primers were used, clearly suggesting that the mutation site is located outside the *xdh* operon and is regulatory in nature. However, without further characterization we cannot exclude the possibility that the regulatory mutation affecting the XDH phenotype may locate somewhere else in the ATCC43072 genome, and that the difference found in the sequences has no relevance to the XDH-positive phenotype.

7.4.3. Production of xylitol-4-dehydrogenase in *E. coli*

Since it proved difficult to purify sufficient amounts of native xylitol-4-dehydrogenase for characterization, the enzyme was produced recombinantly in *E. coli*. The *P. ananatis xdh* gene was cloned into the overexpression vector pKK223-3, resulting in plasmid pKTH5185, and transferred to *E. coli* M15. The *E. coli* clone carrying pKTH5185 was

designated as ERF2157. The XDH enzyme was purified to homogeneity from IPTG induced *E. coli* ERF2157 cells. The molecular mass estimated from the SDS-PAGE gel was 29 kDa, which corresponds well with the molecular mass of 28.0 kDa calculated from the amino acid sequence. The molecular mass of the recombinant enzyme was estimated by analytical gel filtration as 100 kDa, suggesting that the enzyme is either a trimer or a tetramer.

7.4.4. Production of L-xylulose

L-xylulose was produced using resting recombinant *E. coli* ERF2157 cells. Xylitol was efficiently converted to L-xylulose and no major by-products could be detected in the incubation medium by HPLC. Although the results suggest that the recombinant xylitol-4-dehydrogenase also has side-activity for D-xylulose (20% of that for L-xylulose), no evidence of D-xylulose being synthesized by the recombinant cells was found. A similar production experiment has been previously carried out using resting cells of the *P. ananatis* ATCC43074 mutant (Doten & Mortlock 1985a). In this case, analysis of the ketose produced from xylitol also suggested that the D form was absent in the xylulose excreted into the medium. It would, however, seem plausible that the affinity of the enzyme is far greater for L-xylulose than it is for D-xylulose in the concentrations that these ketoses are present in the cell. In addition, *E. coli* is known to metabolize D-xylose by isomerizing it to D-xylulose, which is then phosphorylated to D-xylulose-5-phosphate (Gottschalk 1986). It is also possible that some of the D-xylulose formed could be metabolized *via* this pathway.

In the cell suspension containing initially 5 g/l xylitol, 94% (mol/mol) of the xylitol consumed was converted to L-xylulose after 19 h of incubation and the yield of L-xylulose from the xylitol initially present was 82% (mol/mol). At the initial xylitol concentration of 10 g/l, 89% (mol/mol) of the xylitol consumed was converted to L-xylulose after 19 h and the yield from total xylitol was 70%.

The current production strain compares favorably with the natural L-xylulose producers reported in the literature. The highest L-xylulose yields of 80% for *Alcaligenes* sp. 701B (Khan et al. 1991) and of 70% for *P. ananatis* ATCC43074 (Doten & Mortlock 1985a) from xylitol were obtained in these studies at a xylitol concentration of 5 g/l in 24 h and 18 h, respectively. With the recombinant cells used in the present study a high yield (70%) could also be achieved in 19 h with an initial xylitol concentration of 10 g/l, whereas in the previous reports with *Alcaligenes* sp 701B and *P. ananatis* only 50% and 48% yields, respectively, could be achieved at this xylitol concentration. The possibility of using higher xylitol concentrations enables higher volumetric productivities. Furthermore, it is most likely that optimization of the production conditions (pH, aeration, initial xylitol concentration) would result in even more efficient conversion of xylitol to L-xylulose.

8. CONCLUSIONS AND FUTURE ASPECTS

In this thesis, the main target was to modify LAB metabolism to produce industrially important compounds. During the past decade, the significance of LAB in the production of valuable metabolites has increased and metabolic engineering of LAB has resulted in an efficient production of, for instance, diacetyl and alanine (Hugenholtz et al. 2000, Hols et al. 1999a). However, due to its cost-effectiveness, most of the fine chemicals are currently produced by chemical reactions, even though there are problems associated with the high purity required of starting materials, involuntary side products, often environmentally hazardous reaction components and the lack of steric specificity. The biotechnical production of chemicals by fermentation has become a more attractive alternative as genetic engineering has introduced new product candidates by combining enzymes from different origins into novel production pathways. Furthermore, the possibility of using industrial side streams as raw materials in fermentation processes, based on the intrinsic specificity of enzymatic reactions, could further increase the cost-effectiveness of this alternative. There are also some compounds, including rare sugars such as L-xylulose, for which microbial production appears to be superior to the complicated chemical methods.

In this work, the first part described metabolic engineering to improve the production of mannitol, pyruvate and L-lactic in LAB. The second part described the isolation, cloning and characterization of genes encoding enzymes that are promising for the further development of new genetically modified lactic acid bacteria.

In the first part of the study on *L. fermentum*, which is an efficient mannitol producer, either its *ldhD* gene and *ldhL* gene, or *ldhD* alone, were inactivated by using a gene replacement system. These gene inactivations successfully created two *L. fermentum* mutant strains that produce mannitol and either pure L-lactate or pyruvate in a single process. Moreover, mannitol production by *L. pseudomesenteroides* was improved by using random mutagenization to decrease its fructokinase activity.

In the second part of this work, the mannitol dehydrogenase gene *(mdh)* of *L. mesenteroides* was isolated. This enables further enhancement of mannitol production in *L. mesenteroides* by *mdh* overexpression. The *mdh* gene could also be expressed in other *Lactobacillus* strains possessing otherwise good process capacities. Furthermore, the cloning of a xylitol-4-dehydrogenase gene from the gram-negative *P. ananatis* was accomplished. It is the first available bacterial gene sequence that encodes the enzyme catalyzing the oxidation of xylitol to L-xylulose. The availability of a xylitol-4-dehydrogenase enzyme will now offer an interesting opportunity for its co-expression with xylose reductase in a recombinant LAB strain. Such a recombinant LAB strain could reduce xylose to xylitol by xylose reductase, followed by oxidation of xylitol to L-xylulose and excretion out of the cell. Nyyssölä et al. (2005) have already constructed a strain that expresses the D-xylulose reductase from yeast *Pichia stipitis*. This *L. lactis* recombinant strain converted 34% of xylose to xylitol in the resting state. The accumulated xylitol probably inhibits the reduction reactions, hindering the full conversion of xylose to xylitol (Nyyssölä et al. 2005). The addition of xylitol-4-

dehydrogenase activity in this strain could prevent the accumulation of xylitol and would enable the conversion of D-xylose to valuable L-xylulose.

So far, the metabolic engineering of LAB has mainly been based on deletions or overexpression of the key genes of a particular pathway. The metabolic pathways of *L. lactis*, the model organism of LAB, are well characterized. Much is also known of the multilevel control systems that have evolved to allow *L. lactis* to respond to various environmental challenges. The same complex control network also makes *L. lactis* remarkably recalcitrant to many engineering attempts on its pathways, particularly due to the role of key co-metabolites. Therefore, such approaches have often led to unexpected consequences in the bacterial growth rate or metabolite productions. Presently, new techniques such as microarrays, whole genome sequencing and nuclear magnetic resonance spectroscopy (NMR) are increasing the available information on the genes, gene expressions, genomes and metabolic routes of LAB. In the future, this increasing knowledge will facilitate the construction of quantitative models of metabolic pathways.

Pyruvate branching and glycolysis models are already available for *L. lactis* that have succeeded in predicting modifications in metabolic pathways (Hoefnagel et al. 2002a, b). Hoefnagel et al. (2002a) studied a kinetic model of pyruvate branches in *L. lactis* and experimentally confirmed the predictions of this model. For example, the model predicted that knocking out lactate dehydrogenase and overexpressing NADH oxidase would increase the flux through the acetolactate synthase route.

Some doubts and restrictions have, however, been presented for these *in silico* models and their possibilities to predict and simulate the metabolic pathways in general (Kleerebezem et al. 2002, Smid et al. 2005b). One argument is that to compute a model that could take into account complex interactions between enzymes and intracellular intermediates needs a high amount of computing power. Secondly, the analyses of intermediate metabolites required for the validation of the models would be very time-consuming. Thirdly, many of the kinetic parameters cannot yet be determined because of the low activities of the enzymes and the lack of enzyme assays (Kleerebezem et al. 2002). Even being optimistic, there is still much to do at the basic level, such as analyzing the kinetic properties of the enzymes and measuring the intermediate levels in the cell by NMR. However, development is occurring rapidly and at least the models for the relatively simple pathways will probably facilitate the engineering and optimization of metabolic pathways in LAB in the near future. At present, *L. lactis* is one of the most promising candidates for the systems biology approach and thus predictive models will no doubt emerge.

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